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SUBTITLE: Evaluation of Biomarkers for Sulfur Mustard Exposure in the Euthymic Hairless Mouse Model

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The objective of this study was to investigate the pharmacologic modulation of biomarkers for evaluating therapeutics against HD. The molecular biomarkers investigated, using a ribonuclease protection assay (RPA) to determine messenger ribonucleic acid (mRNA) levels, were mediators of inflammation, including several cytokines and chemokines, tenascin, and ornithine decarboxylase. Biochemical biomarkers investigated were serum amyloid P (SAP), interleukin-6 (IL-6), and interleukin-1 alpha (IL-1α) using ELISAs, and activity of myeloperoxidase (MPX) using a spectrophotometric method. Other endpoints included histopathology and edema measurements. Exposure to HD resulted in a time-dependent increase in mRNA levels of monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-2 (MIP-2), macrophage inflammatory protein-1 alpha (MIP-1α) and interleukin-1 beta (IL-1β). Exposure to HD also resulted in edema and apparent increases in the protein levels of SAP and IL-6, and in the activity of MPX. Four drug treatments, olvanil, dexamethasone, hydrocortisone, and indomethacin, were shown to modulate HD-induced inflammation.

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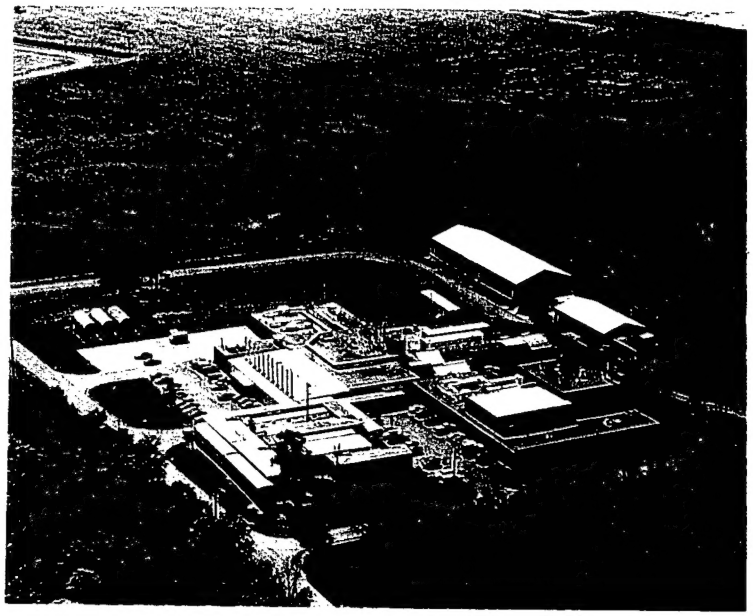

To

U.S. Army Medical Research

Institute of Chemical Defense

November, 2000

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FINAL REPORT

Contract No. DAMD17-89-C-9050
A Medical Research and Evaluation Facility (MREF) and Studies
Supporting the Medical Chemical Defense Program

on

TASK 95-41
EVALUATION OF BIOMARKERS FOR SULFUR MUSTARD EXPOSURE IN THE EUTHYMIC HAIRLESS MOUSE MODEL

to

U.S. ARMY MEDICAL RESEARCH
INSTITUTE OF CHEMICAL DEFENSE

November, 2000

by

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In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources, National Research Council, National Academy Press, Washington, D.C., 1996.

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FINAL REPORT

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U.S. ARMY MEDICAL RESEARCH
INSTITUTE OF CHEMICAL DEFENSE

November 2000

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EXECUTIVE SUMMARY

The research objective of this study for the U. S. Army Medical Research Institute of Chemical Defense (USAMRICD) was to investigate the pharmacologic modulation of biomarkers for evaluating therapeutics against the chemical warfare agent, sulfur mustard (2,2'-dichlorodiethyl sulfide; HD). Medical Research and Evaluation Facility (MREF) Task 95-41, "Evaluation of Biomarkers for Sulfur Mustard Exposure in the Euthymic Hairless Mouse Model", was initiated on July 24, 1996 to identify and evaluate biomarkers indicative of exposure to the vesicating agent, sulfur mustard in the hairless mouse vesicant model (HMVM). The molecular biomarkers investigated using a ribonuclease protection assay (RPA) to determine messenger ribonucleic acid (mRNA) levels, were mediators of inflammation, including several cytokines and chemokines, tenascin, and ornithine decarboxylase (ODC). Biochemical biomarkers investigated were serum amyloid P (SAP), interleukin-6 (IL-6), and interleukin-1 alpha (IL-1α) levels using enzyme-linked immunosorbent assays (ELISA), and activity of the enzyme myeloperoxidase (MPX) using a spectrophotometric method. The effect of topically applied anti-inflammatory drug treatments was determined by quantitating alterations in molecular and biochemical biomarkers in skin following HD challenge. Other endpoints in this study included histopathological evaluations and edema measurements.

Module I investigated the effect of HD exposure on molecular and biochemical biomarkers in the HMVM. Exposure to HD resulted in a time dependent increase in the mRNA levels of monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-2 (MIP-2), macrophage inflammatory protein-1 alpha (MIP-1α) and interleukin-1 beta (IL-1β). Exposure to HD also resulted in edema, as determined by an increase in relative skin weight (RSW). HD exposure also was associated with significant increases in the protein levels of SAP and IL-6, and the activity of MPX.

Module II determined the effect of four drug treatments, ICD 2723 (olvanil; OLV), ICD 2845 (dexamethasone; DEX), ICD 2842 (hydrocortisone; HC), and ICD 2086 (indomethacin; IND), in reducing edema and histopathological markers 24 hr post-exposure, and the efficacy of OLV and DEX in reducing HD-induced increases in molecular and biochemical biomarkers in the HMVM. OLV and DEX decreased HD-mediated inflammation as determined by RSW. OLV decreased the HD-mediated increase in IL-6 protein levels and MPX enzyme activity.

DEX decreased the HD-mediated increase in IL-1β mRNA levels, IL-6 protein levels, and MPX enzyme activity. Histopathologically, HD increased the incidence of epidermal necrosis (EN) and had no effect on follicular necrosis (FN), intracellular edema (IE), microvesication (MV), and pustular epidermitis (PE). Drug treatment had no effect on EN.

Prior to the completion of Modules I and II, the study was expanded to include a third Module, conducted in the mouse ear vesicant model (MEVM). In Module III, HC, DEX, and IND treatments were effective in moderating HD-induced inflammation, as determined by relative ear weights (REW). HC and DEX reduced REW at 6 hr and IND reduced REW at 6 and 24 hr. Treatment was effective in reducing HD-mediated increases in MCP-1, MIP-2, and IL-1β mRNA levels. HC and OLV reduced the HD-mediated increase in MIP-2 mRNA levels at 2 hr; IND reduced the HD-mediated increase in MIP-2 mRNA levels at 2 and at 24 hr; HC and DEX reduced the HD-mediated increase in IL-1β mRNA levels at 6 hr; and DEX reduced the HD-mediated increase in MCP-1 mRNA levels at 6 h

TASK 95-41

EVALUATION OF BIOMARKERS FOR SULFUR MUSTARD EXPOSURE IN THE EUTHYMIC HAIRLESS MOUSE MODEL

1.0 INTRODUCTION

Sulfur mustard (2,2'-dichlorodiethyl sulfide; HD) is a chemical warfare agent that penetrates the skin rapidly and in man causes extensive blistering after a latent period of several hours (Papirmeister *et al.*, 1991). Cutaneous exposure to HD results in inflammation, epithelial tissue damage, and damage to the anchoring complex of the epidermal-dermal junction, including the formation of microscopic subepidermal blisters (Papirmeister *et al.*, 1991). Similarly, in animal models, the histopathologic changes occurring within 24 hr of exposure include erythema, edema, microscopic subepidermal blisters, and epidermal necrosis (Smith *et al.*, 1997; Casillas *et al.*, 1997a).

The use or threat of use of HD in military conflict warrants the study of biomarkers of HD exposure. Currently, there are no established prophylactic or therapeutic countermeasures against HD-induced skin injury. The U. S. Army Medical Research Institute of Chemical Defense (USAMRICD) has a mission to evaluate candidate topical and systemic therapeutics against HD-induced injury. Battelle's Medical Research and Evaluation Facility (MREF) Task 95-41, "Evaluation of Biomarkers for Sulfur Mustard Exposure in the Euthymic Hairless Mouse Model", was initiated on July 24, 1996 to identify and quantitate biomarkers of HD challenge and to measure the pharmacologic modulation of these biomarkers in animal models. The biomarkers investigated are known to play major roles in acute and chronic inflammation.

The Hairless Mouse Vesicant Model (HMVM) and the Mouse Ear Vesicant Model (MEVM) were developed to provide a qualitative measure of the inflammatory response following HD exposure by determining skin edema. Acute dermal inflammation is known to involve a complex network of interactions between mediators that influence, and are influenced by, one another. Cytokines play an important role in regulating localized inflammatory responses. Biomarkers of HD exposure recently have been identified in the MEVM (Sabourin

and Casillas, 1998; Ricketts *et al.*, in press; Sabourin and Casillas, in press). These inflammatory mediators include granulocyte macrophage-colony stimulating factor (GM-CSF), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α) and macrophage inflammatory protein-1 alpha (MIP-1α). These studies investigated the temporal relationship between cytokine gene expression and the pathobiological response in mouse skin exposed to HD using quantitative reverse transcription-polymerase chain reaction, enzyme-linked immunosorbent assay (ELISA) and immunohistochemistry. Exposure to HD resulted in an increase in IL-1β, TNF-α, and MIP-1α messenger ribonucleic acid (mRNA) levels at 3 hr. All inflammatory mediators showed increased mRNA levels at 6-24 hr following exposure. Immunohistochemistry localized the cytokine proteins to different cell types within the mouse skin. GM-CSF was localized in inflammatory cells independent of post-exposure time. IL-1β and IL-6 immunostaining was observed at multiple sites within the skin, and increased intensity of staining was first observed at 6 hr following exposure. Exposure to HD resulted in a time dependent increase in IL-6 protein levels in skin in the MEVM and in the HMVM. Skin interleukin-1 alpha (IL-1α) protein was increased post-exposure to HD in the HMVM, however IL-1α mRNA and protein levels were not increased following HD exposure in the MEVM. These studies identified increases in the *in vivo* expression of inflammatory cytokines as early as 3 hr following HD exposure, which precedes the HD-induced histopathological damage (necrosis, subepidermal blister) known to occur after 12 hr in these models, and provided the ground work for Task 95-41.

Task 95-41 consisted of three modules. Module I investigated molecular and biochemical biomarkers in the HMVM at 2, 6, and 24 hr post-exposure. Module II and Module III investigated the effect of topically administered OLV, DEX, HC, and IND in reversing the HD-induced alterations in the biomarkers in the HMVM at 24 hr and in the MEVM at 2, 6, and 24 hr. OLV is classified pharmacologically as a vanilloid, and is an analog of capsaicin, the pungent principle in *Capsicum* peppers. A role for vanilloids as mediators of skin inflammation is the depletion of substance P and other neuropeptides with subsequent effects like the blockade of vascular leakage (Veronesi *et al.*, 1995). The glucocorticoids, DEX and HC are anti-inflammatory agents known to block cytokine production. IND is a non-steroidal anti-inflammatory compound and a cyclooxygenase inhibitor.

The molecular biomarkers investigated in Modules I and II included interleukin-12 p35 (IL-12 p35), interleukin-12 p40 (IL-12 p40), interleukin-10 (IL-10), IL-1α, IL-1β, interleukin-1 receptor antagonist (IL-1RA), macrophage migration inhibitory factor (MIF), IL-6, interferon-γ (IFN-γ), interleukin-4 (IL-4), interleukin-11 (IL-11), TNF-α, GM-CSF, macrophage inflammatory protein-2 (MIP-2), MIP-1α, interferon-γ-inducible protein-10 (IP-10), MCP-1, transforming growth factor-beta (TGF-β), regulated upon activation-normal T-cell expressed and secreted (RANTES), eotaxin, tenascin, ODC, and two housekeeping genes, glyceraldehyde phosphate dehydrogenase (GAPDH) and L32. The molecular biomarkers investigated in Module III included IL-1β, RANTES, eotaxin, IL-6, MIP-1α, MIP-2, MCP-1, tenascin, ODC, L32 and GAPDH.

Biochemical endpoints examined included the inflammatory cytokine proteins IL-1α and IL-6, previously examined in the HMVM (Ricketts *et al.*, in press), serum amyloid P (SAP) and myeloperoxidase (MPX). SAP is a major acute phase reactive protein in mice and is released by hepatic tissue in response to cytokines released from cells at the site of injury. MPX is found in high concentration in the granules of polymorphonuclear cells (PMN) and has been shown to be quantitatively related to the number of PMN in tissue (Bradley *et al.*, 1982). PMN rapidly infiltrate the site of injury and induce respiratory burst activity and lysosomal enzyme release. MPX activity has been shown to increase in the skin of HD-exposed hairless guinea pigs (Bongiovanni *et al.*, 1993).

Histopathological evaluation has been used widely to measure HD-induced dermal injury, but, in part because it is a time consuming, subjective process, there has been interest from the chemical defense research community to investigate the potential of molecular and biochemical biomarkers as adjunct endpoints. The biomarker results of Task 95-41 can be used to evaluate the effectiveness of medical countermeasures in the HMVM and MEVM.

2.0 MATERIALS AND METHODS

2.1 Animal Models

Male, euthymic hairless mice (Cr1:SKH1-hrBR), used for Modules I and II, were obtained from Charles River Laboratories (Portage, MI). Animal husbandry and manipulations for SKH1 hairless mice were performed under Protocol No. 118 (Appendix A) at the MREF, a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International).

The animal model used for Module III was the CD1 mouse. Animal husbandry procedures and dosing of CD1 mice were performed at the USAMRICD.

2.2 HD Exposure and Drug Application

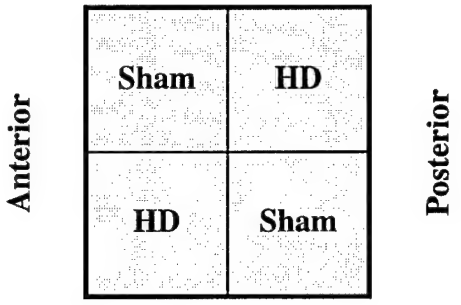
2.2.1 Hairless Mouse Vesicant Model

Modules I and II were conducted using the HMVM. Mice were weighed and anesthetized with a combination of ketamine and xylazine administered intraperitoneally. Each animal was secured in sternal recumbency by taping the legs and tail to a 4″ by 6″ cardboard pad. Vapor cap units (14-mm diameter polypropylene cap with a Whatman™ Paper #2 disc inserted on the inside of the cap) were saturated with 10 µL HD (d = 1.27 g/mL; MW 159). Vapor cap units for sham sites received no HD. Vapor caps were secured to the dorsum of the animal for 6 min; forceps were used to apply or remove the vapor cap units. Of the four sites, two positioned on either side of the dorsal midline, two were sham and two were exposed to HD with or without treatment. After HD exposure, mice were maintained in polycarbonate cages for the duration of the experiment. Each cage was covered with a plastic-backed paper pad to protect the animals from drafts. Animals were euthanatized by Halothane® overdose. Following sacrifice, skin was immediately excised from the dorsum, and a 12-mm diameter biopsy punch was used to remove full-thickness skin samples from experimental sites. Samples to be used for Ribonuclease Protection Assay (RPA) analysis were snap-frozen in liquid nitrogen and stored at –70 C.

Samples to be used for biochemical analyses were weighed, snap-frozen in liquid nitrogen, and stored at –70 C.

Module I. There were 4 experimental sites per animal, positioned on either side of the dorsal midline according to the following schematic:

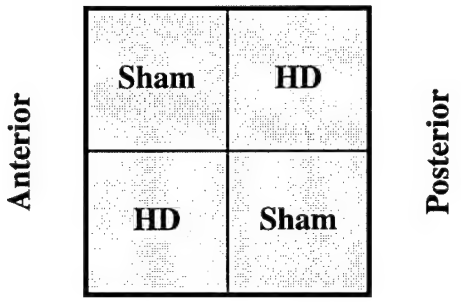
Dorsal Surface of Animal



Module II. There were 4 experimental sites per animal, positioned on either side of the dorsal midline. The following schematics were used for histopathology, RPA, and biochemical analyses.

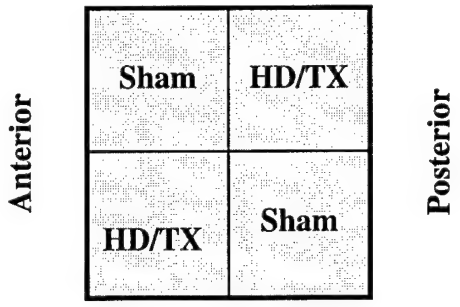
HD Control Group - Histopathological Analyses

Dorsal Surface of Animal



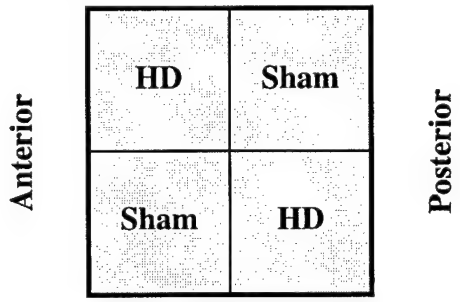
Drug Treatment Groups - Histopathological Analyses

Dorsal Surface of Animal



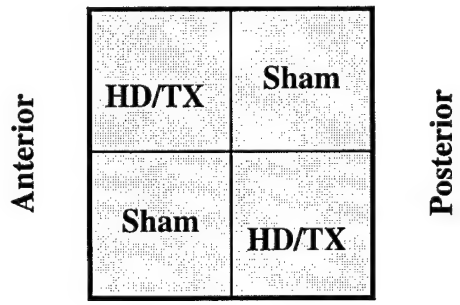
HD Control Group - Biochemical Biomarker Analyses

Dorsal Surface of Animal



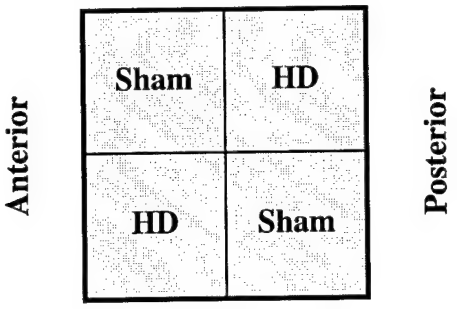
Drug Treatment Groups - Biochemical Biomarker Analyses

Dorsal Surface of Animal



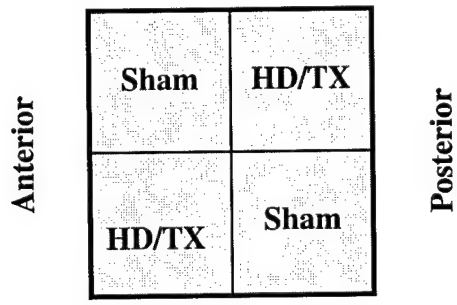
HD Control Group - RPA Biomarker Analyses

Dorsal Surface of Animal



Drug Treatment Groups - RPA Biomarker Analyses

Dorsal Surface of Animal



Drug treatments used in Module II included OLV (0.25 mg/application), HC (0.25 mg/application), IND (1 mg/application) and DEX (0.5 mg/application), applied to the appropriate sites at –2, +2, and +6 hr with respect to HD exposure.

2.2.2 Mouse Ear Vesicant Model

Module III was conducted using the MEVM. Experimental procedures related to sample generation were conducted at USAMRICD. Anesthetized mice placed in dorsal recumbency were exposed topically to liquid HD (0.16 mg) in dichloromethane on the medial surface of the right ear using published procedures (Casillas *et al.*, 1997b). Animals were euthanatized at 2, 6, or 24 hr following HD exposure. The left ear was not treated. In the HD Control groups, the

right ears received 10 µL of ethanol 15 min prior to HD. In the drug treatment groups, the right ears received treatments as follows: OLV (0.25 mg/application) applied –15 min with respect to HD exposure, DEX (0.5 mg/application) applied –2 hr with respect to HD exposure, HC (0.25 mg/application) applied –2 hr with respect to HD exposure, and IND (1 mg/application) applied –15 min with respect to HD exposure. After euthanasia, an 8-mm diameter biopsy sample was taken from each ear, snap-frozen in liquid nitrogen, and sent to the MREF on dry ice.

2.3 Histopathology

Module II studies included histopathological analyses. Tissues were fixed in 10 percent neutral buffered formalin (NBF). Fixed specimens were embedded in paraffin, sectioned, and stained with hematoxylin and eosin for evaluation. Tissues were scored for microscopic subepidermal blisters, follicular necrosis, intracellular edema, epidermal necrosis, and pustular epidermitis. Tissue was ranked with a severity score when a histopathologic marker was evident within the affected tissue. The degree of severity ranged in value from 1 through 4. The scores indicated the following:

- "1" – lesion involves <5 percent of the section;
- "2" – lesion involves 10 to 40 percent of the section;
- "3" – lesion involves 50 to 80 percent of the section; and
- "4" – lesion involves >90 percent of the section.

A score of "0" indicated no marker was present.

2.4 RPA

RPA was performed as detailed in MREF Method Nos. 49 and 54/*In Vitro* (Appendix C, D) using RNA isolated from tissue samples. Frozen samples placed in RNAzol™ reagent (Tel-Test; Friendswood, TX) were homogenized using a Tekmar-Dohrman Tissuemizer™ (Cincinnati, OH). After chloroform extraction, RNA was precipitated in isopropanol, washed with ethanol, suspended in a nuclease-free solution, and stored at approximately -70 C. The quality of the RNA was evaluated by agarose gel electrophoresis and ethidium bromide staining.

Three custom RPA panels (Pharmingen, San Diego, CA), each representing 11 genes of interest, were used for analyses. Each panel consisted of RNA templates available for T7 polymerase-directed *in vitro* transcription into a high specific activity [α-^{32}P]-labeled (NEN Life Science Products, Boston, MA) antisense probe. Radiolabeled antisense RNA probe was incubated with RNA isolated from tissue specimens for approximately 16 hr to form double-stranded RNA:RNA hybrids. Any remaining single-stranded RNA was treated enzymatically with ribonuclease A and ribonuclease T1. The undigested RNA:RNA hybrids were treated with proteinase K, extracted with chloroform, and precipitated in ethanol (Eastman Kodak Co., New Haven, CT). After washing with ethanol, hybridized RNAs were dissolved in gel loading buffer, heat-denatured, and placed on ice prior to denaturing polyacrylamide gel electrophoresis. Vacuum-dried gels were exposed overnight at -20 C using X-AR film and an intensifying screen (Eastman Kodak Co., New Haven, CT) to produce autoradiographs for densitometric analysis.

2.4.1 Data Analysis: RPA Analyses

Autoradiographs were scanned with a Bio Rad GS-700 imaging densitometer (Bio Rad, Hercules, CA) and analyzed with Multi Analyst software (Bio Rad, Hercules, CA). For each sample, volume densities were calculated, expressed as the optical density x mm^2, for specific bands compiling the gene expression profile. Naïve samples were evaluated under identical experimental conditions concurrently with samples from HD-exposed and/or HD plus drug treatment samples. Data were standardized and expressed as a fraction of vehicle control response. The fractional response was calculated by dividing the densitometry value for the HD-exposed samples by the average value for the vehicle control samples at the same time point. Standard Error (SE) bars are included in the graphs.

2.5 Preparation of Skin Homogenates

Snap-frozen specimens were processed using a liquid nitrogen-cooled Biopulverizer (Daigger, Lincolnshire, IL). Pulverized samples were solubilized in 1X phosphate buffered saline (PBS), and centrifuged at 50,000 x g for 30 minutes, as detailed in MREF Method No. 55/*In vitro* (Appendix E). The supernatants were decanted for protein, IL-1α, IL-6 and SAP analyses, and the pellets were used for MPX measurements.

2.6 MPX Enzyme Activity Assay

Samples were prepared for MPX analysis as detailed in MREF Method No. 55/*In vitro* (Appendix E). Pellets were removed from –70 C storage and 0.5 percent hexadecyltrimethyl ammonium bromide (HTAB) solution (Sigma, St. Louis, MO) was added. Samples were briefly sonicated (Model 450, Branson Inc., Danbury, CT), subjected to three freeze/thaw cycles using liquid nitrogen, and sonicated again. Samples were centrifuged at 50,000 x g for 30 minutes at 4 C. The supernatant was stored at -70 C. MPX was analyzed following published methodology (Bradley *et al.*, 1982). Standards, isolated from a purified human PMN MPX preparation, (Calbiochem, La Jolla, CA) and samples were added to wells of 96-well microplates (VWR, So. Plainfield, NJ). The reaction was started by adding 20 µL of sample to 180 µL of 50 mM potassium phosphate solution containing 0.00025 percent hydrogen peroxide and 0.19 mg/mL *o*-dianisidine (Sigma, St. Louis, MO) under reduced lighting conditions. Absorbance at 450 nm was measured using a microplate reader (THERMOmax model; SoftMaxPro software, Molecular Devices, Sunnyvale, CA). Activity level (U/mL) in samples was calculated by regression analysis. Results are expressed as units of activity/mg tissue.

2.7 Protein Determinations

Protein concentrations were determined using the bicinchoninic acid (BCA) Method (Pierce Chemical Co., Rockford, IL) standardized with a commercial preparation of bovine serum albumin (Pierce Chemical Co.), as detailed in MREF Method No. 22/*In vitro* (Appendix B). Replicate protein samples were analyzed and the results averaged. Results are expressed as mg/mL.

2.8 SAP Measurements

SAP was measured using a sandwich ELISA technique, as detailed in MREF Method No. 56/*In vitro* (Appendix F) and essentially as described by Burlingame, *et al.*, 1996. SAP standards (Calbiochem, La Jolla, CA) were utilized. Affinity-purified sheep anti-mouse SAP antibody (Calbiochem) was coated onto Immulon II ELISA plates (Corning, Cambridge, MA). The remaining protein binding sites were saturated with 0.1 percent gelatin blocking buffer (Sigma, St. Louis, MO). After the blocking step, microplates were incubated and washed. The secondary antibody, rabbit anti-mouse SAP (Calbiochem) was added and allowed to bind at room temperature. Horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma) was then added. For visualization, 1-Step-Turbo (3,3′,5,5′-, tetramethyl-benzidine) TMB Enzyme Immunoassay Reagent (Endogen, Woburn, MA) was added under reduced lighting conditions. Reactions were stopped by the addition of dilute sulfuric acid (Mallincrodt Chemicals, St. Louis, MO). Absorbance at 450 nm was measured using a microplate reader (THERMOmax model; SoftMaxPro software, Molecular Devices, Sunnyvale, CA). SAP levels (ng/mL) in samples were calculated by regression analysis. Results are expressed as ng/mg protein/mg tissue.

2.9 Mouse IL-6 ELISA

IL-6 ELISA were performed according to the manufacturer's instructions (Quantikine mouse, R & D Systems, Minneapolis, MN). IL-6 concentration (pg/mL) in samples was determined by regression analysis. Results are expressed as pg/mg protein/mg tissue.

2.10 Mouse IL-1α ELISA

IL-1α ELISA were performed according to the manufacturer's instructions (Endogen, Woburn, MA). IL-1α concentration (pg/mL) in samples was calculated by regression analysis. Results are expressed as pg/mg protein/mg tissue.

2.11 Data Analysis: Histopathology, Edema, and Biochemical Markers

Methods for data analyses of histopathology and relative tissue weights are located in Appendices G-K.

3.0 RESULTS AND CONCLUSIONS

3.1 Module I

Module I studied the effect of HD on relative tissue weight, and on alterations in mRNA levels of inflammatory mediators, (SAP, IL-6, IL -1α protein), and MPX enzyme activity, at 2, 6, and 24 hr post-exposure in the HMVM. Four sites were on the back of each animal. Of the four sites, two positioned on either side of the dorsal midline, two were sham and two were exposed to HD. Eight or four animals were at each post-exposure time for biochemical analyses or for RPA analyses, respectively. The average response of the two HD sites or the two sham sites within each animal was used as the endpoint for statistical analysis.

3.1.1 RSW

The edema response was determined from weighed 12-mm diameter skin biopsy samples taken from the center of HD-exposed and sham-exposed sites (Table 1). There was no increase in RSW at 2 hr post-exposure. For HD-exposed sites, at 6 and 24 hr, RSW was significantly increased compared to sham sites. RSW at 24 hr was significantly different than at 2 and 6 hr.

Table 1. RSW in the HMVM

Time Post-Exposure (hr)	Percent RSW Mean ± SE
2	0.65 ± 2.19[1]
6	9.19 ± 3.98[1]
24	33.9 ± 4.88

[1]Significantly different from the 24 hr group ($p < 0.05$).

3.1.2 Molecular Biomarkers

RPA was used to measure the effect of HD on the mRNA levels of 24 genes in the HMVM at 2, 6, or 24 hr. The mRNA levels for MCP-1, MIP-2, MIP-1α, and IL-1β showed a time dependent up-regulation with exposure to HD (Figure 1). TNF-α was increased at 6 hr but returned to control levels by 24 hr. IL-1α, IL-11, IL-1RA, MIF, and TGF-β were detectable but showed no alteration in expression following HD exposure. Expression of GM-CSF, IFN-γ, IL-12 p35, IL-12 p40, IL-10, IL-4, IL-6, and IP-10 was not detected.

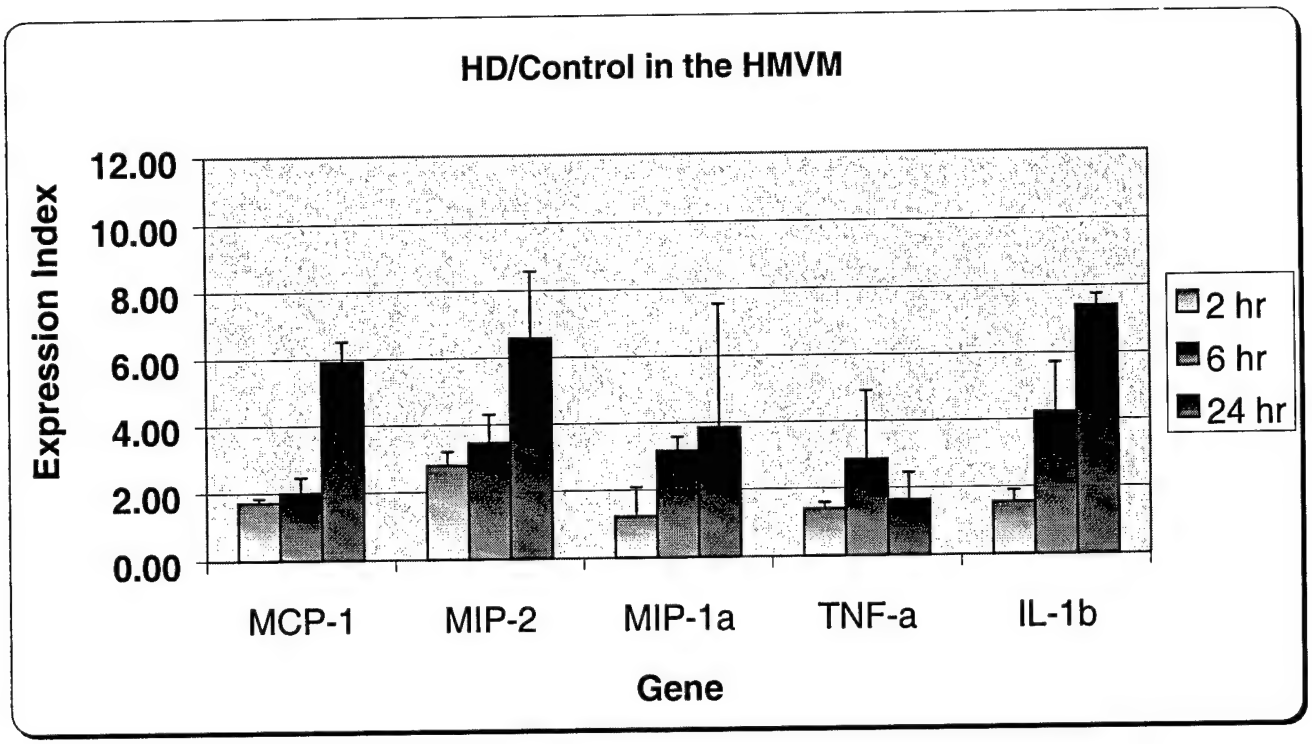


Figure 1. Gene Expression of Inflammatory Mediators in the HMVM at 2, 6, and 24 hr Post-Exposure to HD.

The graphs show the ratio of the means of the HD-exposed to the control sites. The SE are shown.

3.1.3 Biochemical Biomarkers

SAP protein levels were increased with HD exposure at 2 and 6 hr post-exposure, however the increase was not significant (Table 2). SAP protein levels were significantly increased in HD-exposed sites at 24 hr.

Table 2. SAP Protein Levels in the HMVM

Time Post-Exposure (hr)	SAP (ng/mg protein/mg tissue) Mean ± SD	
	Sham	HD
2	0.066 ± 0.067	0.083 ± 0.088
6	0.086 ± 0.040	0.091 ± 0.042
24	0.169 ± 0.173	0.312 ± 0.318[1]

[1]Significantly different from sham ($p < 0.001$).

IL-6 protein levels were significantly increased in the HD-exposed sites at 6 hr (Table 3). IL-6 protein levels also were increased following HD exposure at 2 and 24 hr, however, the increase was not statistically significant.

Table 3. IL-6 Protein Levels in the HMVM

Time Post-Exposure (hr)	IL-6 (pg/mg protein/mg tissue) Mean ± SD	
	Sham	HD
2	0.030 ± 0.032	0.049 ± 0.052
6	0.133 ± 0.248	1.100 ± 0.751[1]
24	0.048 ± 0.044	0.194 ± 0.089

[1]Significantly different from sham ($p < 0.001$).

MPX levels were significantly increased in the HD-exposed sites at 24 hr (Table 4).

Table 4. MPX Activity in the HMVM

Time Post-Exposure (hr)	MPX (U/mg) Mean ± SD	
	Sham	HD
2	0.138 ± 0.063	0.119 ± 0.055
6	0.159 ± 0.097	0.194 ± 0.055
24	0.155 ± 0.058	0.339 ± 0.138[1]

[1]Significantly different from sham ($p < 0.001$).

IL-1α protein levels were significantly decreased in the HD-exposed sites at 24 hr (Tables 5). IL-1α protein levels were not altered at 2 and 6 hr post-exposure.

Table 5. IL-1α Protein Levels in the HMVM

Time Post-Exposure (hr)	IL-1α (pg/mg/mg) Mean ± SD	
	Sham	HD
2	6.959 ± 1.084	7.480 ± 2.172
6	8.729 ± 2.756	7.845 ± 2.622
24	8.520 ± 3.722	4.069 ± 1.241[1]

[1]Significantly different from sham at ($p < 0.001$).

3.1.4 Module I Conclusions

Edema response, as evaluated by RSW, showed a time dependent increase over the 24 hr time course. Cutaneous exposure for 6 min to HD vapor in the HMVM produced an inflammatory response, as determined by quantitative molecular and biochemical techniques. Exposure to HD was associated with a time dependent increase in the mRNA levels of MCP-1,

MIP-2, MIP-1α, and IL-1β over the 24 hr evaluated. TNF-α mRNA levels were increased at 6 hr. Exposure to HD was associated with significant increases in SAP protein levels at 24 hr, IL-6 protein levels at 6 hr post-exposure, and MPX enzyme activity at 24 hr post-exposure. In Module I, IL-1α protein levels decreased at 24 hr post-exposure, therefore IL-1α was not evaluated in Module II.

3.2 Module II

Module II determined the efficacy of four drug treatments, OLV, DEX, HC, and IND in reducing RSW at 24 hr post-exposure, and the efficacy of OLV and DEX in reducing molecular and biochemical biomarker response at 24 hr post-exposure in the HMVM. Two studies were performed in Module II. Animals in Study 1 were examined for RSW and histopathology with all four drug treatments. Animals in Study 2 were examined for RSW and for molecular and biochemical biomarkers following OLV and DEX treatment.

3.2.1 RSW

Study 1.

RSW significantly decreased with OLV, HC, and IND treatment (Table 6). Statistical analyses are in Appendix K

Table 6. RSW with Drug Treatment in the HMVM – Study 1

Group	Percent RSW Mean ± SE	Percent Reduction from HD Control
HD CONTROL	33.0 ± 4.6	-
OLV	14.4 ±5.4[1]	56
DEX	20.8 ± 6.8	37
HC	11.7 ± 5.1[1]	64
IND	7.6 ± 3.8[1]	77

[1]Significantly different from HD group ($p < 0.05$).

Study 2.

Drug treatments significantly reduced the RSW (Table 7). The percent reduction from the HD Control group was approximately 50 percent. Statistical analyses are in Appendix K

Table 7. RSW with Drug Treatment in the HMVM – Study 2

Group	Percent RSW Mean ± SD	Percent Reduction from HD Control
HD CONTROL	44.4 ± 5.7	-
OLV	20.6 ±7.9[1]	54
DEX	23.4 ± 5.0[1]	47

[1]Significantly different from HD group ($p < 0.05$).

3.2.2 Histopathology

Study 1.

Histopathological evaluations were performed to determine drug efficacy in reducing EN, FN, IE, MV, and PE in HD-exposed sites. Statistical analyses are in Appendix J. Statistically, DEX reduced the incidence of MV and PE, and HC reduced the incidence of PE, however a reduction from means of less than 1 as observed in the HD Control group are not relevant for analysis (Table 8). Therefore, the statistical analysis of the histopathologic markers did not reflect an alteration in the HMVM.

Table 8. Histopathology Markers in HD-Exposed Sites in the HMVM

Group		EN	FN	IE	MV	PE
HD CONTROL	Mean ± SD	3.25 ± 0.93	1.44 ± 0.73	0	0.94 ± 0.57	0.38 ± 0.50
	Percent Incidence	100	88	0	81	38
OLV	Mean ± SD	3.20 ± 0.95	1.50 ±0.76	0	0.90 ±0.79	0.25 ±0.44
	Percent Incidence	100	90	0	70	25
DEX	Mean ± SD	2.50 ± 1.10	1.20 ±0.77	0.05 ±0.22	0.35 ±0.49	0.05 ±0.22
	Percent Incidence	95	80	5	35[1]	5[1]
HC	Mean ± SD	3.00 ± 1.08	1.60 ±0.68	0	0.70 ±0.73	0
	Percent Incidence	100	95	0	55	0[1]
IND	Mean ± SD	2.90 ± 1.17	1.30 ±0.86	0.20 ± 0.41	1.05 ±0.83	0.25 ± 0.44
	Percent Incidence	95	80	20	75	25

3.2.3 Molecular Biomarkers

In the HMVM, DEX and OLV reduced the HD-mediated increase in IL-1β gene expression at 24 hr; OLV did not alter the HD-induced gene expression of tenascin, MCP-1, MIP-2 (Figure 2).

The graph shows the mean from six animals normalized to the expression of the housekeeping gene, GAPDH. The SE are shown.

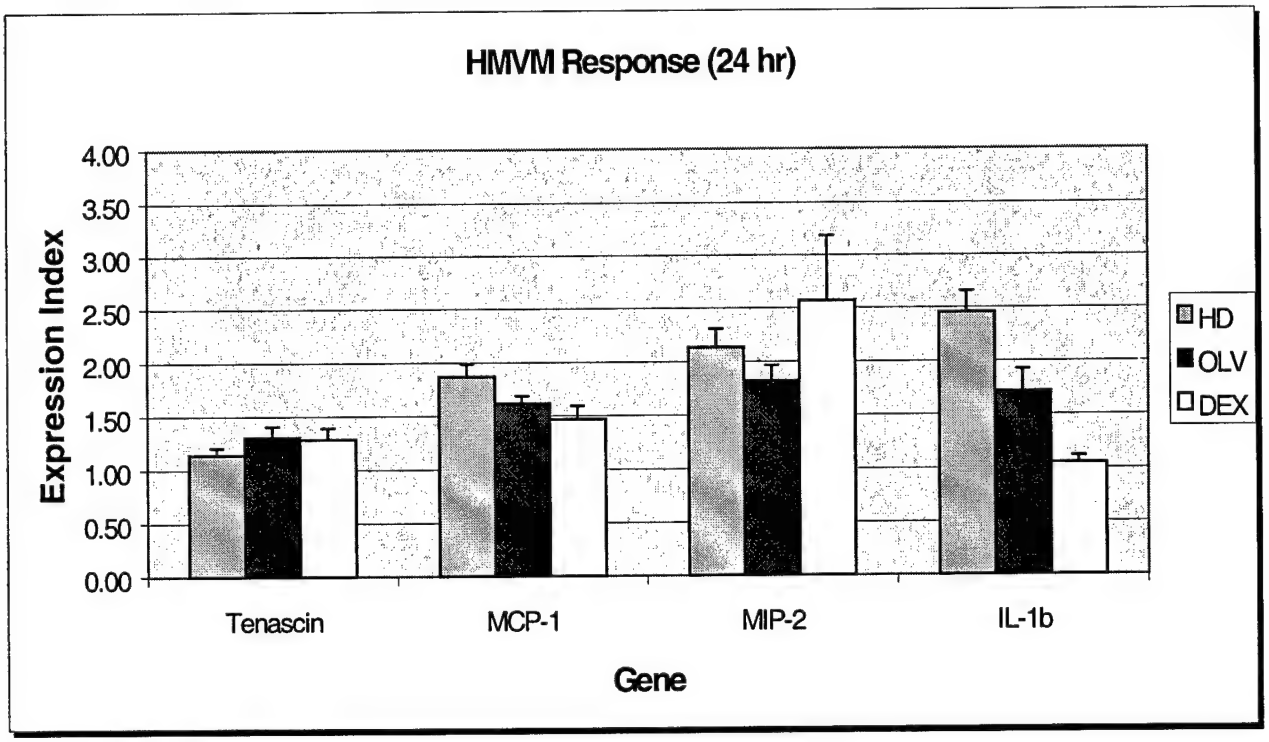


Figure 2. Alteration in the Expression of Inflammatory Mediators in the HMVM with OLV and DEX Treatment at 24 hr Post-Exposure to HD.

3.2.4 Biochemical Markers

SAP levels in the control group were increased with HD exposure, however the increase was not significant (Table 9). There was no reduction in SAP levels with drug treatment. Statistical analyses are in Appendix H.

Table 9. SAP Protein Levels with Drug Treatment in the HMVM

Group	SAP (ng/mg protein/mg tissue) Mean ± SD	
	Sham	Exposed
HD CONTROL	0.251 ± 0.223	0.435 ± 0.187
OLV	0.515 ± 0.484	0.635 ± 0.518
DEX	0.331 ± 0.164	0.757 ± 0.377[1]

[1]Significantly different from sham ($p < 0.001$).

IL-6 protein levels were significantly increased with HD-exposure in the control group (Table 10). IL-6 levels were reduced with drug treatment. Statistical analyses are in Appendix H.

Table 10. IL-6 Protein Levels with Drug Treatment in the HMVM

Group	IL-6 (pg/mg protein/mg tissue) Mean ± SD	
	Sham	Exposed
HD CONTROL	0	0.616 ± 0.553[1]
OLV	0.115 ± 0.189	0.239 ± 0.099[2]
DEX	0.089 ± 0.192	0.075 ± 0.139[2]

[1]Significantly different from sham ($p < 0.001$).
[2]Significantly different from the HD CONTROL group; OLV group ($p = 0.026$) a and DEX group ($p = 0.002$).

MPX activity levels were significantly increased with HD-exposure in the control group (Table 11). OLV and DEX resulted in a significant reduction in the HD-induced response for MPX. Statistical analyses are in Appendix H.

Table 11. MPX Activity with Drug Treatment in the HMVM

Group	MPX (U/mg tissue) Mean ± SD	
	Sham	Exposed
HD CONTROL	0.464 ± 0.224	0.802 ± 0.232[1]
OLV	0.423 ± 0.141	0.496 ± 0.157[2]
DEX	0.142 ± 0.069	0.198 ± 0.090[2]

[1] Significantly different from sham ($p< 0.001$).
[2] Significantly different from HD CONTROL group; OLV group ($p< 0.001$) and DEX group ($p < 0.001$).

3.2.5 Module II Conclusions

Alterations in biomarkers with drug treatment in the HMVM at 24 hr are summarized in Table 12. Drug treatment with OLV, DEX, HC, and IND significantly decreased HD-mediated inflammation. OLV decreased IL-6 protein and MPX enzyme activity. DEX decreased IL-1β mRNA levels, IL-6 protein, and MPX enzyme activity. In contrast to the significant increase in SAP levels observed at 24 hr post-exposure to HD in Module I, an increase was not observed in Module II, and therefore SAP could not be used to evaluate drug efficacy.

Table 12. Alterations in Biomarkers with Drug Treatment in the HMVM at 24 hr

Marker	OLV	DEX	HC	IND
RSW	↓	↓	↓	↓
Tenascin mRNA	No effect	No effect	ND	ND
MCP-1 mRNA	No effect	No effect	ND	ND
MIP-2 mRNA	No effect	No effect	No effect	No effect
MIP-1α mRNA	No effect	No effect	No effect	No effect
IL-1β mRNA	↓	↓	ND	ND
SAP protein	*	*	ND	ND
IL-6 protein	↓	↓	ND	ND
MPX enzyme activity	↓	↓	ND	ND

↓Results indicate a significant decrease in HD-mediated biomarker.
*Results indicate there was not a statistically significant increase in SAP in the HD-exposed sites compared to the control sites.
ND – Not done.

3.3 Module III

Module III was conducted to determine the efficacy of four drug treatments, IND, OLV, HC, and DEX, at 6 and 24 hr following exposure to HD using the MEVM. The right ear was treated with IND, OLV, HC, DEX, or ethanol as a control treatment, and exposed to HD. The left ear was not treated. At each exposure time there were 10 animals in each treatment group for biochemical analyses, 6 for RPA, and 5 control animals. The endpoints evaluated were relative ear weight (REW), molecular biomarkers, and the biochemical biomarkers, SAP, IL-6, and MPX. Descriptive statistics for REW, SAP, IL-6, and MPX at 6 and 24 hr post-exposure are in Appendix I.

3.3.1 REW

IND, HC, and DEX significantly decreased REW at 6 hr, however, at 24 hr post-exposure, only IND significantly reduced REW (Table 13). Statistical analyses are in Appendix I.

Table 13. REW with Drug Treatment in the MEVM

Time Post-Exposure (hr)	Group	Percent REW Mean ± SE	Percent Reduction
6	HD CONTROL	57.8 ± 9.8	-
	IND	24.7 ± 6.5[1]	57
	OLV	50.5 ± 7.1	13
	HC	13.4 ±1.8[1]	77
	DEX	12.4 ± 1.0[1]	79
24	HD CONTROL	158 ± 5.0	-
	IND	102 ± 10.4[1]	36
	OLV	132 ± 15.9	17
	HC	136 ± 12.1	14
	DEX	143 ± 11.6	10

[1]Significantly different from HD CONTROL group ($p < 0.05$)

3.3.2 Molecular Biomarkers

RPA was used to determine mRNA levels for IL-1β, RANTES, eotaxin, IL-6, MIP-1α, MIP-2, MCP-1, tenascin, and ODC in the MEVM (Figure 3). Transcripts for RANTES, eotaxin, IL-6, and MIP-1α were not detected. The mRNA levels of ODC showed a time dependent increase over the 24 hr time course. MCP-1 and IL-1β mRNA levels were increased at 6 hr but returned to control levels at 24 hr. MIP-2 mRNA levels increased at 6 hr and remained elevated at 24 hr. Tenascin mRNA levels were not altered with HD exposure.

Figure 3 shows the mean from six animals normalized to the expression of the housekeeping gene, GAPDH. The SE are shown.

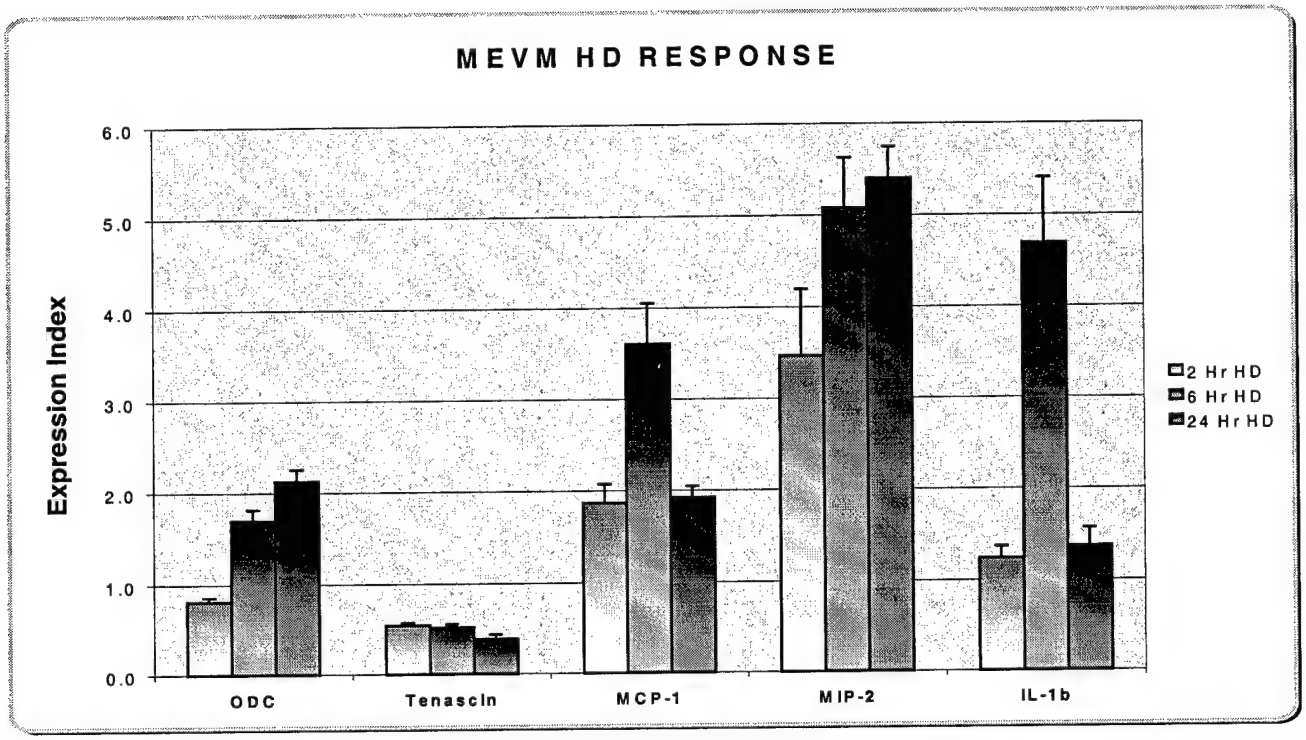


Figure 3. Expression of ODC, Tenascin, MCP-1, MIP-2, and IL-1β mRNA in the MEVM at 2, 6, and 24 hr Following Exposure to HD.

The effect of drug treatment on the HD-induced mRNA levels was evaluated in the MEVM at 2, 6, and 24 hr following exposure to HD. At 2 hr, HC, IND, and OLV decreased the HD-mediated increase in MIP-2 mRNA levels (Figure 4A). At 6 hr, HC and DEX decreased the HD-mediated IL-1β mRNA increase (Figure 4B). At 24 hr, IND decreased the HD-mediated increase in MIP-2 mRNA levels (Figure 4C).

The graphs show means from six animals normalized to the expression of the housekeeping gene, GAPDH. The SE are shown.

Figure 4A

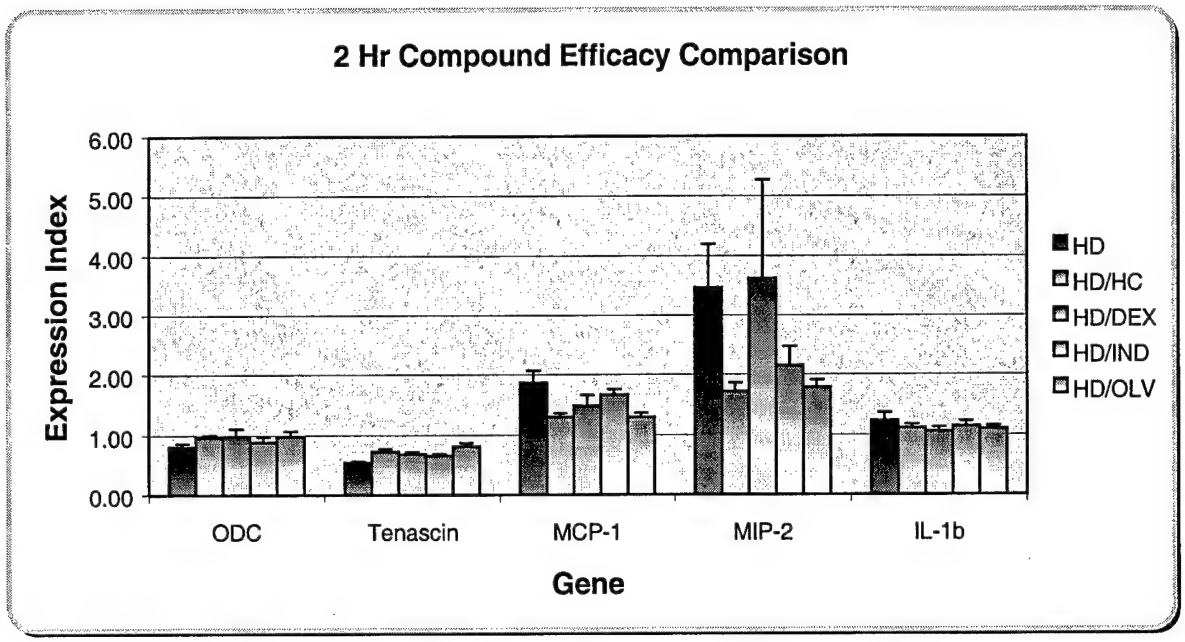


Figure 4B

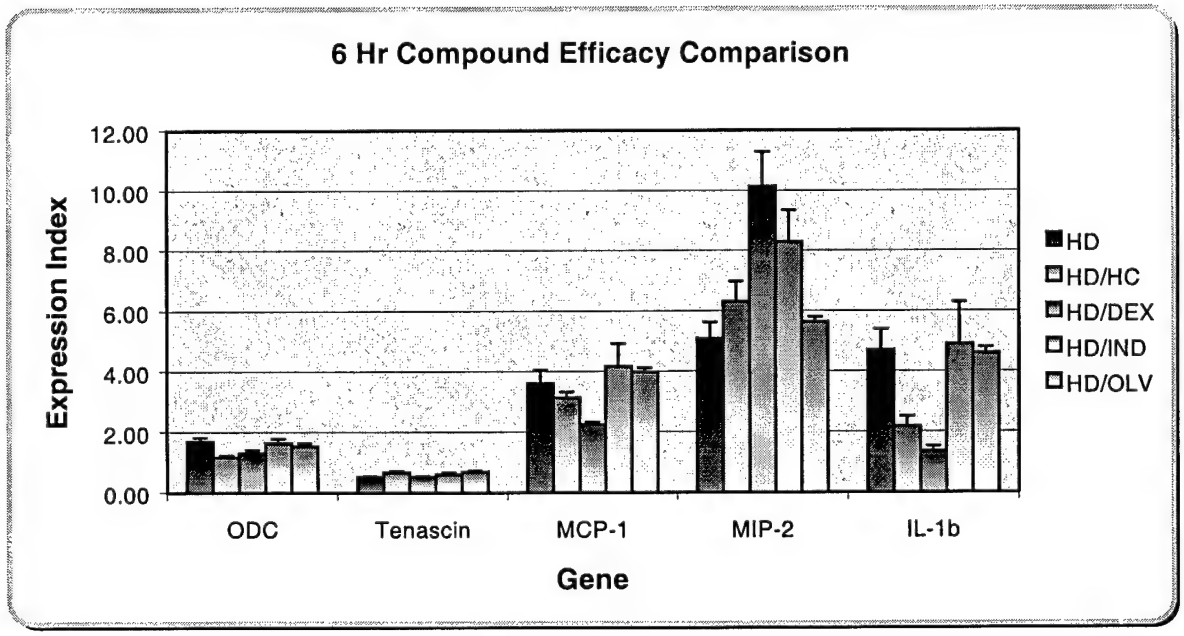
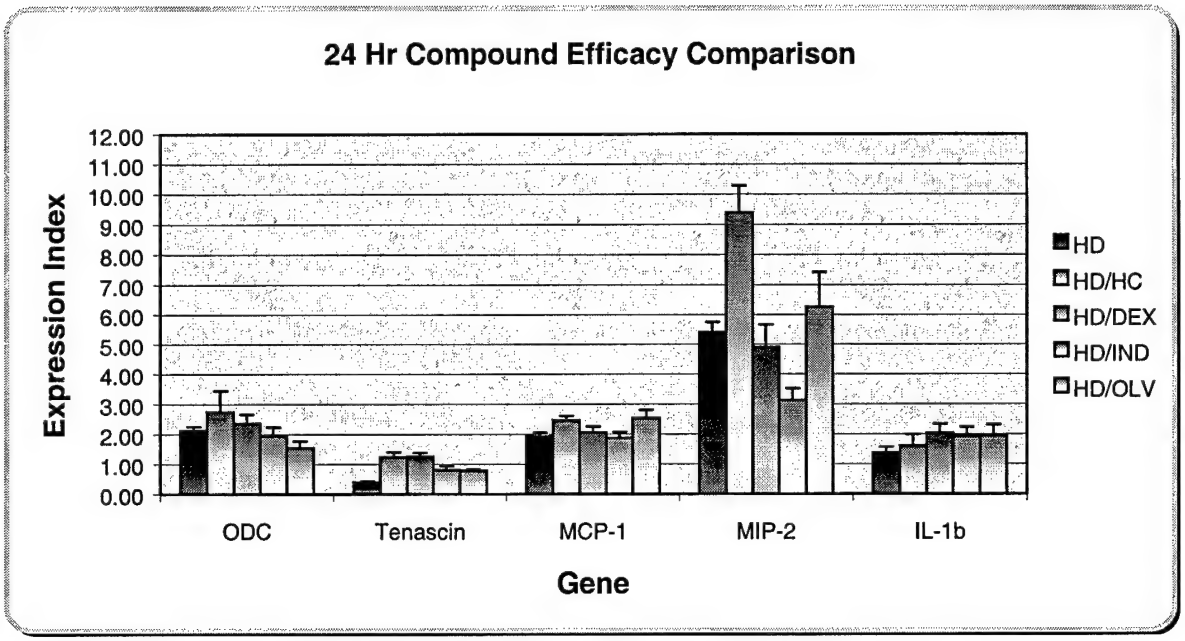


Figure 4C



Figures 4 (A, B, C). Expression of Inflammatory Mediator mRNA in the MEVM with HC, DEX, IND, and OLV at 2 (A), 6 (B), and 24 (C) hr Post-Exposure to HD.

3.3.3 Biochemical Biomarkers.

In the MEVM, SAP protein levels were increased with HD exposure at 6 hr in the control group, however the increase was not statistically significant. No difference in SAP protein levels was observed at 24 hr (Table 14). Statistical analyses are in Appendix I.

Table 14. SAP Protein Levels with Drug Treatment in the MEVM

Time Post-Exposure (hr)	Group	SAP (ng/mg protein/mg tissue) Mean ± SD	
		Control Ear	HD Exposed Ear
6	HD CONTROL	0.432 ± 0.142	1.304 ± 0.732
	IND	1.262 ± 0.825	2.295 ± 1.447
	OLV	1.176 ± 1.484	1.959 ± 1.547
	HC	2.877 ± 1.131	3.343 ± 2.574
	DEX	2.021 ± 1.548	3.332 ± 2.060[1]
24	HD CONTROL	13.228 ± 22.306	14.489 ± 6.688
	IND	2.737 ± 1.321	38.341 ± 22.270[1, 2]
	OLV	2.049 ± 1.138	7.463 ± 9.776
	HC	2.820 ± 3.852	5.344 ± 3.230
	DEX	1.813 ± 0.991	6.053 ± 6.104

[1] Significantly different from control ear at 6 hr, IND ($p = 0.028$), and DEX ($p = 0.004$) and at 24 hr, IND group ($p < 0.001$).
[2] Significantly different from HD CONTROL group at 24 hr; IND group ($p < 0.001$).

IL-6 protein levels at 6 and 24 hr post-exposure were not increased with exposure to HD in control animals (Table 15) and therefore the efficacy of drug treatment could not be evaluated. Statistical analyses are in Appendix I.

Table 15. IL-6 Protein Levels with Drug Treatment in the MEVM

Time Post-Exposure (hr)	Group	IL-6 (pg/mg protein/mg tissue) Mean ± SD	
		Control Ear	HD Exposed Ear
6	HD CONTROL	2.081 ± 2.172	1.180 ± 0.661
	IND	1.622 ± 0.701	2.620 ± 1.193
	OLV	2.476 ± 1.392	7.735 ± 2.535[1, 2]
	HC	8.245 ± 3.201	9.603 ± 4.523
	DEX	9.644 ± 2.008	7.883 ± 2.717[1]
24	HD CONTROL	3.069 ± 3.290	0.725 ± 0.368
	IND	0	0.887 ± 1.301
	OLV	0	0
	HC	0	0.700 ± 0.620
	DEX	0	0

[1] Significantly different from control ear at 6 hr, OLV ($p < 0.001$), and DEX ($p = 0.041$).
[2] Significantly different from HD CONTROL group at 6 hr; IND group ($p < 0.001$).

MPX activity levels were increased with HD exposure at 6 hr, however the increase was not significant (Table 16). There was no increase in MPX levels at 24 hr with HD exposure. Therefore, the efficacy of drug treatment could not be evaluated at either time point. Statistical analyses are in Appendix I.

Table 16. MPX Enzyme Activity with Drug Treatment in the MEVM

Time Post-Exposure (hr)	Group	MPX (U/mg tissue) Mean ± SD	
		Control Ear	HD Exposed Ear
6	HD CONTROL	0.459 ± 0.286	0.769 ± 0.136
	IND	0.631 ± 0.533	1.599 ± 0.717[1]
	OLV	0.830 ± 0.573	1.549 ± 0.513[1]
	HC	0.417 ± 0.269	1.251 ± 0.659[1]
	DEX	0.597 ± 0.411	0.937 ± 0.306
24	HD CONTROL	2.251 ± 4.200	1.160 ± 0.313[1, 2]
	IND	0.496 ± 0.204	1.810 ± 0.942[1, 2]
	OLV	0.578 ± 0.471	1.865 ± 0.688[1, 2]
	HC	0.362 ± 0.147	1.724 ± 0.648[1, 2]
	DEX	0.169 ± 0.122	1.227 ±0.472[1, 2]

[1]Significantly different from control ear at 6 hr, IND ($p < 0.001$), OLV ($p < 0.001$), and HC ($p < 0.001$) and 24 hr, IND ($p = 0.007$), OLV ($p = 0.009$), HC ($p = 0.006$), and DEX ($p = 0.029$).
[2]Significantly different from HD CONTROL group; IND ($p = 0.015$), OLV ($p = 0.017$), HC ($p = 0.013$), and DEX ($p = 0.033$).

3.3.4 Module III Conclusions

In the MEVM, MCP-1 mRNA levels were maximally elevated at 6 hr; MIP-2 mRNA levels were elevated at 2, 6, and 24 hr; and IL-1β mRNA levels were elevated at 6 hr only. HD exposure did not have a statistically significant effect on SAP protein, IL-6 protein, or MPX activity.

Alterations in REW and molecular biomarkers in the MEVM with drug treatment are summarized in Table 17. Drug treatments were effective in moderating HD-induced inflammation as assessed by REW. IND, HC, and DEX significantly decreased REW at 6 hr, however, at 24 hr post-exposure, only IND significantly reduced REW. Drug treatment was efficacious in reducing mRNA levels of MCP-1, MIP-2, and IL-1β. HC, IND, and OLV reduced

MIP-2 mRNA levels at 2 hr; HC and DEX reduced IL-1β mRNA levels at 6 hr; and DEX reduced MCP-1 mRNA levels at 6 hr.

Table 17. Alterations in Biomarkers with Drug Treatment in the MEVM

Marker	HC			DEX			IND			OLV		
	2 hr	6 hr	24 hr	2 hr	6 hr	24 hr	2 hr	6 hr	24 hr	2 hr	6 hr	24 hr
REW	ND	↓		ND	↓		ND	↓	↓	ND		
MCP-1 mRNA					↓							
MIP-2 mRNA	↓						↓		↓	↓		
IL-1β mRNA		↓			↓							

↓ Results indicate a significant decrease in HD mediated increase in marker by the drug.
ND – Not done.

4.0 DISCUSSION

Task 95-41 was initiated to evaluate alterations in molecular and biochemical mediators of inflammation in the HMVM and MEVM through evaluation of the expression of inflammatory mediators at the mRNA and protein level, and the enzyme activity of MPX. RPA was used to determine the mRNA levels of cytokines, chemokines, tenascin, and ODC. In the HMVM, MCP-1, MIP-2, MIP-1α, IL-1β, and TNF-α showed a time dependent up-regulation with exposure to HD. In the MEVM, MCP-1, MIP-2, and IL-1β mRNA levels increased with exposure to HD. ELISA analyses identified increases in the protein levels of SAP and IL-6, and MPX enzyme activity with exposure to HD in the HMVM.

Because histopathological damage, known to occur after 12 hr following HD exposure in the MEVM and in the HMVM (Smith *et al.*, 1997; Casillas *et al.*, 1997b), is preceded by an inflammatory response, there would seem to be a window of opportunity for pharmacologic intervention. Histopathology studies in the HMVM highlight the subjectivity of these

evaluations and the need for biomarkers to quantitatively assess HD damage and treatment efficacy. In this study, treatment with DEX significantly lowered the incidence of microscopic subepidermal blisters and pustular epidermitis compared to HD-exposed controls, and similarly, HC significantly lowered the incidence of pustular epidermitis compared to HD-exposed controls. However, the decreases in MV and PE in the drug treatment groups were from an initial value of < 1 in the HD control group which indicates an involvement of < 5 percent of the section.

Results from the molecular studies demonstrated drug treatment modulation in decreasing the inflammatory response in both models. In the HMVM, DEX decreased HD-mediated increases in IL-1β mRNA levels, IL-6 protein levels and MPX enzyme activity. In the MEVM, there was a dramatic reduction in HD-mediated MIP-2 gene expression in HD-exposed samples that had been treated with HC, OLV or IND. In addition, DEX decreased the level of HD-induced MCP-1 mRNA, and HC and DEX treatment decreased the HD-mediated increase in IL-1β mRNA.

This study identified biomarkers useful for studying the inflammatory response in HD-induced cutaneous injury and demonstrated a modulation in these biomarkers with drug treatment. The use of these biomarkers in drug treatment studies contributes to the continued development of quantifiable *in vivo* biological markers of HD injury to evaluate the effectiveness of medical countermeasures.

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APPENDIX A

MREF Protocol 118 and Amendments

Evaluation of Biomarkers for Sulfur Mustard Exposure in the Euthymic Hairless Mouse Model

Study Performed by Battelle Memorial Institute,
Medical Research and Evaluation Facility
505 King Avenue, Building JM-3
Columbus, Ohio 43201-2693

STUDY TITLE:

Evaluation of Biomarkers for Sulfur Mustard Exposure in the Euthymic Hairless Mouse Model

PRINCIPAL INVESTIGATOR:

James A. Blank, Ph.D., Study Director — Date 7/23/96

SCIENTIFIC REVIEW:

John B. Johnson, D.V.M., M.S. — Date 7/23/96
Manager and Co-Principal Investigator
Medical Research and Evaluation Facility

Carl T. Olson, D.V.M., Ph.D. — Date 7/24/96

ATTENDING/CONSULTING VETERINARIAN:

Tracy A. Peace, D.V.M., Study Veterinarian — Date 072596

STATISTICAL REVIEW:

Ronald G. Menton, Ph.D., Study Statistician — Date 7/23/96

CONTRACTING OFFICER'S REPRESENTATIVE:

LTC Richard R. Stotts, D.V.M., Ph.D. — Date 25 Jul 96
U.S. Army Medical Research Institute of Chemical Defense
(USAMRICD)

PROTOCOL TITLE: Evaluation of Biomarkers for Sulfur Mustard Exposure in the Euthymic Hairless Mouse Model

PRINCIPAL INVESTIGATOR: James A. Blank, Ph.D., Study Director

CO-INVESTIGATOR(S):

Study Supervisor: Laurie A. Lane

Statistician: Ronald G. Menton, Ph.D.

Study Veterinarian: Tracy A. Peace, D.V.M.

Study Pathologist: Allen W. Singer, D.V.M.

Study Chemist: Timothy L. Hayes, B.A.

Sponsor: U.S. Army Medical Research Institute of Chemical Defense (USAMRICD)

Sponsor Monitor: LTC Richard R. Stotts, D.V.M., Ph.D., Contracting Officer's Representative (COR), USAMRICD

I. NON-TECHNICAL SYNOPSIS:

The use or threat of use of Sulfur mustard, [bis(2-chloroethyl) sulfide; HD] in military conflicts has stressed the need for the development of antivesicant compounds, and the characterization of a suitable animal model for screening potential prophylactic and therapeutic compounds to prevent or treat vesicant agent injury. The objective of studies described in this protocol are to screen skin and blood samples for biomarkers that may be predictive of cutaneous HD vapor exposure.

II. BACKGROUND:

A. Background:

HD is capable of producing incapacitating injury to the skin of exposed individuals. The hallmark of HD-induced skin damage in humans is the formation of large fluid-filled blisters which tend to heal slowly (Eisenmenger et al., 1991). Animal models which exhibit frank blisters in response to HD have not been identified, however the skin of euthymic hairless mice and euthymic hairless guinea pig have been shown to exhibit microvesication (limited epidermal-dermal separation) and

these animals have been used to investigate potential mechanisms of HD-induced skin damage, as well as to develop preventative and therapeutic strategies to counter HD exposure (Mershon et al., 1990; Marlow et al., 1990; Yourick et al., 1992; Braue et al., 1992; CPT R. Casillas - personal communications, 1995-1996). The primary index of HD-induced dermal damage used to assess countermeasure efficacy is histopathology, a process which is semi-quantitative and time consuming. Parameters such as nicotinamide adenine dinucleotide (NAD^+) concentration, cell-associated protease activity, cell proliferation, and cell viability have been shown to be altered by HD with *in vitro* systems (Meier et al., 1987; Cowan et al., 1991; Smith et al., 1990). *In vivo* studies have shown NAD^+ to be depressed (Gross et al., 1985; Yourick et al., 1992) and interleukin 6 (IL-6) to be elevated (Casillas et al., 1996a) in HD-exposed skin. A biomarker such as IL-6 which provides a quantitative measure would decrease the length of time required for test results and would provide a more sensitive and reliable indicator of damage which would lead to a reduction in animal utilization.

B. Literature Search:

1. Literature Source(s) Searched:

   a. MedLine
   b. Federal Research in Progress (FEDRIP)
   c. Defense Technical Information Center (DTIC)

2. Date and Number of Search:

   a. Medline: 1991-1995
   b. FEDRIP: 1995
   c. DTIC: Inception through 1995

3. Key Words of Search:

   a. Medline: Sulfur Mustard
   b. FEDRIP: Sulfur Mustard
   c. DTIC: Sulfur Mustard and Mice

4. Results of Searches:

   a. Medline: The words "sulfur mustard" had 118 references listed. Examination of this material did not indicate that work performed in this protocol would represent a duplicating of effort.

b. FEDRIP: This search provided one reference for "sulfur mustard". This reference was not relevant to the work outlined in this protocol.

c. DTIC: Nineteen references pertaining to "sulfur mustard" were listed from this database. One reference (Bongiovanni et al., 1993), involved examining skin myeloperoxidase activity following hairless guinea pig exposure to HD. This is different than the work in this protocol, in that mice are being used, and studies to access the predictiveness of HD injury will be performed.

III. OBJECTIVE/HYPOTHESIS: The objective of studies described in this protocol is to screen skin and blood samples for biomarkers that may be predictive of cutaneous HD vapor exposure. Identification of an endpoint suitably predictive of HD-induced skin damage would be of benefit in screening candidate antivesicant compounds and potentially useful in the field for casualty management.

IV. MILITARY RELEVANCE: The use or threat of use of HD in military conflicts has stressed the need for the development of antivesicant compounds, and the characterization of a suitable animal model for screening potential prophylactic and therapeutic compounds to prevent or treat vesicant agent injury. The need for such a model is expressed in the requirements of Joint Service Agreement (JSA) S-A-301 (Prophylactic Drugs), S-A-302 (Antidotes), S-A-303 (CW and BW Therapeutic Drugs), and C-A-303 (Pretreatment Models), and in STO A objectives.

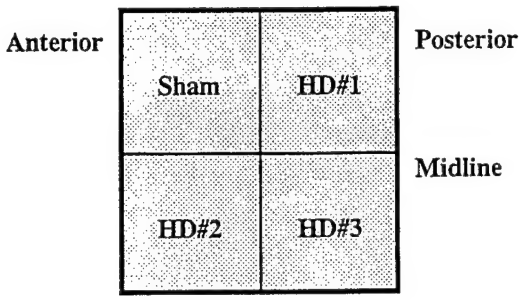
V. MATERIALS AND METHODS:

A. Experimental Design:

1. HD Vapor Exposure Duration-Response Studies: Studies performed at USAMRICD with euthymic hairless mice have defined the relationship between the length of HD vapor exposure and the incidence of microvesication. To transition the model to Battelle, studies will be conducted to confirm the length of HD vapor exposure required to produce a high incidence of microvesication. A HD vapor exposure producing a high incidence of microvesication is needed for subsequent biomarker analyses. Data supplied by USAMRICD will be used to the maximum extent possible to guide the conduct of this study. HD exposure studies are performed on two days, with the results from the first exposure day being used to establish exposure durations for the second exposure day.

a. On the first day, four dosing sites are delineated on the dorsum of each of four mice and erythema measurements are taken by light reflectance using a Minolta Chroma Meter. The animals are anesthetized and exposure sites are exposed to HD vapor for three durations. The vesicating vapor exposure

duration provided by USAMRICD is the HD#2 exposure length indicated in the following illustration. A shorter and a longer exposure duration are also utilized. The dose sites are randomized from animal to animal to prevent experimental bias due to potential positional effects.



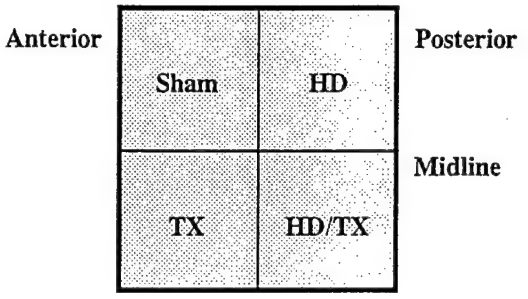
b. Approximately 24 hr following exposure, erythema is assessed. Following euthanasia, edema measurements are made and the tissue sections are placed into neutral buffered formalin for histologic processing and evaluation.

c. Following receipt of the histopathology analysis, HD vapor exposure durations may be selected for the second test day. Ten animals are exposed to HD vapor for three durations using the same animal exposure design as before. Erythema, edema, and histopathologic changes are evaluated as before.

2. Candidate Pre- and Post-Treatment (TX) Compound Evaluations: Transition studies to demonstrate or confirm the effectiveness of four TX compounds are performed. These four TX compounds will have been shown to provide effectiveness against HD-induced edema and/or pathology in the mouse ear model (Casillas et al., 1996b). These compounds are evaluated in the hairless mouse model for effectiveness against HD-induced dermal damage. The histopathologic results from these studies will be used to make comparisons with the same TX compounds on HD-induced biomarker alteration (Module II evaluations). Up to 16 additional animals may be exposed to HD vapors (two sites per animal) for analyses of skin HD content. For these studies, animals are euthanatized at approximately 2 to 4 hr following HD exposure, skin is dissected and extracted in organic solvent. Concentration of extractable HD is then determined.

a. These evaluation studies will utilize the fixed HD vapor duration that had been shown previously to produce a high incidence of microvesication. Ten animals per TX are evaluated for changes in erythema, edema, and

histopathology at approximately 24 hr following HD exposure. TX and HD exposures are applied to the animals as indicated in the following schematic:



b. The dosing sites are randomized from animal to animal to minimize experimental bias due to positional effects. All four TXs are evaluated concurrently, with animals from each group being exposed on each of three days. Erythema, edema, and histopathology measurements are made for each dose site.

3. Endpoint Optimization: The methodology required for each parameter, as well as the amount of tissue needed to make a measurement, is evaluated in this section. Biomarker analyses to be optimized may include those for IL-1 alpha, IL-6, myeloperoxidase (MPX), and serum amyloid P (SAP). Up to 16 animals may be exposed to HD vapor to provide skin and serum samples for the conduct of these studies. Exposures are performed as in Module I evaluations.

   a. If not already known, the sensitivity of the procedure will be defined. Skin homogenate and serum samples are evaluated for the presence of material that may interfere with endpoint measurement. In this evaluation, sample preparations are spiked with known amounts of standards and sample recoveries are determined. Three different spiked samples (e.g., spiked with a low, mid, and high standard) are examined and compared to nonspiked samples.

   b. Sample Volume Determinations - Skin samples which have been snap frozen and stored at approximately -70° C with the serum samples are analyzed. Two tissue sections will be ground in a small volume of buffer (e.g., 1 mL) and the level of biomarker assessed. Undiluted serum specimens are also evaluated. Two-fold serial dilutions of the tissue and serum samples are evaluated. The dilutions of control and HD-exposed skin samples required to provide a good measurement are defined. Similarly, the dilution of serum from naive and HD-exposed animals required to provide a measurable response is determined. This information will minimize the number of biomarker analyses required in later studies.

4. Module I Evaluations: Biological Endpoint Screening: Endpoints are screened for potential utility by performing evaluations at an early (~2 to 4 hr) and later (~24 hr) time following exposure to an HD vapor causing a high incidence of microvesication. Histopathologic measurements are not made in these evaluations. Biomarker responses in serum and tissue samples may be evaluated using four groups of animals as shown in the following table:

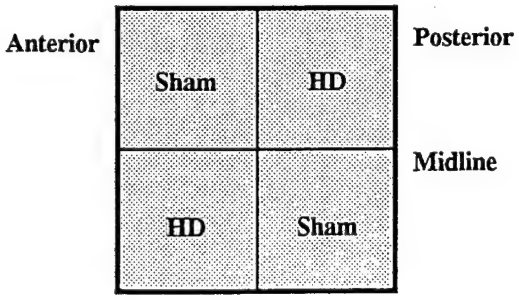
Group	Group Description	N[A]	Sample Time/Source 4 HR	24 HR
A	Naive	8	Plasma & Skin	
B	HD-Exposed	8	Plasma & Skin	
C	Naive	8		Plasma & Skin
D	HD-Exposed	8		Plasma & Skin

[A] Number of animals per group.

a. HD exposures are performed over a two day period with half the animals of each experimental treatment group being exposed on each of two dosing days.

b. Dosing grids are delineated on the dorsa of the animals and the vapor cap assemblies are put in place. Baseline erythema measurements are taken. Animals are anesthetized with ketamine and xylazine, and positioned on a surface (e.g., surgical board, cardboard, etc).

c. Animals from Groups A and C receive four sham HD exposures per animal, while animals in Groups B and D are exposed to HD vapor as indicated in the following schematic.



d. At the indicated times post exposure, erythema is evaluated. The animals are then anesthetized and blood specimens are collected by cardiac puncture

and placed into serum tubes. Following euthanasia, tissue samples are taken and edema measurements performed as described = a later section of this protocol. The tissue specimens are snap-frozen in liquid nitrogen and stored with the serum specimens at approximately - 70° C for biomarker analysis. No histopathology is performed on the skin specimens.

e. If HD does not cause significant ($p < 0.05$) alteration of an endpoint relative to control samples, then the endpoint is not tested further. If HD does cause a significant ($p < 0.05$) response alteration, then the endpoint may be evaluated through Modules II and/or III as directed by the COR.

5. Module II Evaluations: Dermal Biomarker Qualification: The biomarkers and time assessment parameters for Module II evaluations are determined from Module I results. If an HD-induced alteration is observed only at 24 hr, then the 4 hr evaluations are not performed. If an HD-induced alteration is observed only at 4 hr, then the 24 hr time point is not evaluated. If the endpoints are altered at both time points, then Module II assessments may be made at both times, as directed by the COR.

TX compounds that have been shown to be effective in the hairless mouse model are used for Module II evaluations. Up to four TX compounds may be evaluated with skin preparations being analyzed for biomarkers.

Group	Group Description	N	4 Hr Measurements	24 Hr Measurements
A	HD	8	Erythema, Edema, Biomarker	
B	HD/TX#1	8	Erythema, Edema, Biomarker	
C	HD/TX#2	8	Erythema, Edema, Biomarker	
D	HD/TX#3	8	Erythema, Edema, Biomarker	
E	HD/TX#4	8	Erythema, Edema, Biomarker	
F	HD/TX#1	3	Erythema, Edema, PCR[A]	
G	HD/TX#2	3	Erythema, Edema, PCR	
H	HD/TX#3	3	Erythema, Edema, PCR	
I	HD/TX#4	3	Erythema, Edema, PCR	
J	HD	8		Erythema, Edema, Biomarker
K	HD/TX#1	8		Erythema, Edema, Biomarker
L	HD/TX#2	8		Erythema, Edema, Biomarker
M	HD/TX#3	8		Erythema, Edema, Biomarker
N	HD/TX#4	8		Erythema, Edema, Biomarker

[A] Polymerase chain reaction.

a. HD exposures for Groups A to I are performed over a four day period with animals from each group being exposed on each dosing day. HD exposures for Groups J to N are performed over three days with animals from each group being exposed on each dosing day.

b. Dosing grids are delineated on the dorsa of the animals and the vapor cap assemblies are put in place. Baseline erythema measurements are taken. Animals are anesthetized with ketamine and xylazine, and positioned on a surface (e.g., surgical board, cardboard, etc).

c. The dosing schematic for the various groups are presented in the following illustrations:

Groups A and J

Anterior		
	Sham	HD
	HD	Sham

Groups B,C,D,E,K,L,M,

TX	HD/TX
HD/TX	TX

Posterior

Midline

The following dosing schematic is used on the groups exposed to collect skin specimens for PCR analyses.

Groups F,G,H,I

Anterior		
	Sham	HD
	TX Sham	HD/TX

Posterior

Midline

d. At the indicated times post exposure, erythema is evaluated. The animals are euthanatized, tissue samples are taken, and edema measurements performed. The tissue specimens are snap-frozen in liquid nitrogen and

stored at approximately -70° C for biomarker analysis. No histopathology is performed in this module.

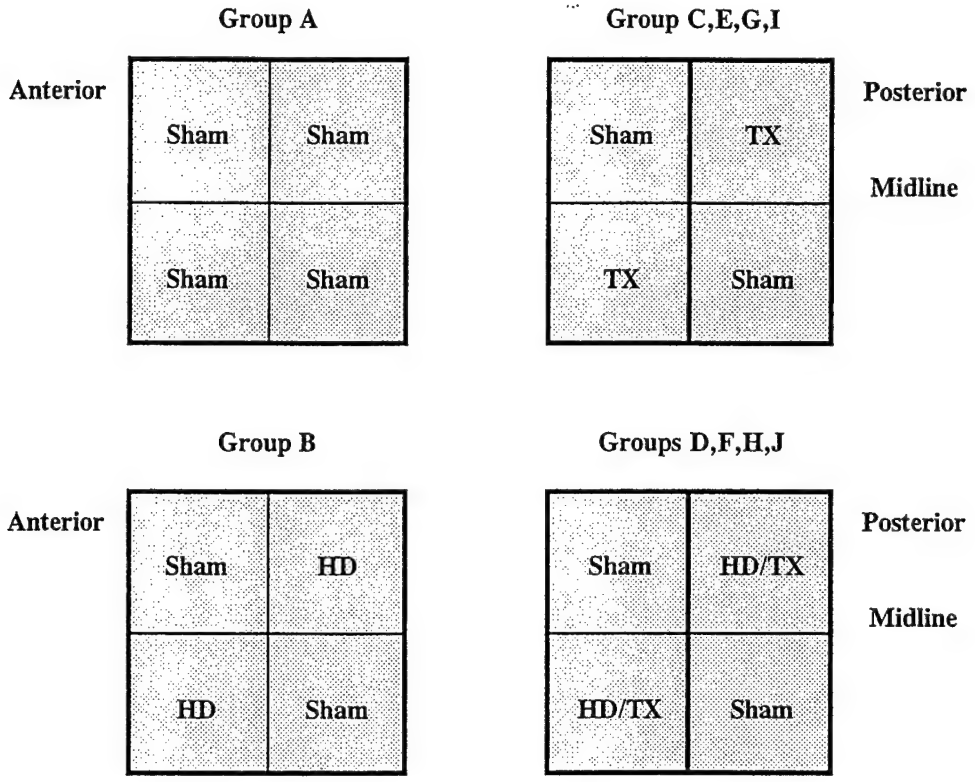
6. Module III Evaluations: Serum Biomarker Qualification: If a biomarker in the serum samples was altered significantly ($p < 0.05$) within 4 hr of HD exposure in Module I evaluations, then the biomarker may be examined in naive animals, treated animals, HD-exposed animals, and HD-exposed animals that have been given a TX. These evaluations are performed only on blood samples collected at the early (~4 hr) time point unless otherwise directed by the COR.

GROUP	Group Description	N	4 Hr Measurements
A	Naive	8	Erythema, Edema, plasma biomarker
B	HD	8	Erythema, Edema, plasma biomarker
C	TX#1	8	Erythema, Edema, plasma biomarker
D	HD+TX#1	8	Erythema, Edema, plasma biomarker
E	TX#2	8	Erythema, Edema, plasma biomarker
F	HD+TX#2	8	Erythema, Edema, plasma biomarker
G	TX#3	8	Erythema, Edema, plasma biomarker
H	HD+TX#3	8	Erythema, Edema, plasma biomarker
I	TX#4	8	Erythema, Edema, plasma biomarker
J	HD+TX#4	8	Erythema, Edema, plasma biomarker

a. Each experimental group consists of eight animals. The exposures may be performed over an eight day period with an animal per treatment group being exposed each day.

b. Dosing grids are delineated on the dorsa of the animals and the vapor cap assemblies are put in place. Baseline erythema measurements are taken. Animals are anesthetized with ketamine and xylazine, and positioned on a surface (e.g., surgical board, cardboard, etc).

c. The dosing schematics for the various groups are presented in the following illustrations:



d. At the indicated times post exposure, erythema is evaluated. The animals are then anesthetized and blood specimens are collected into serum tubes. Following euthanasia, tissue samples are taken and edema measurements are performed. The serum specimens are stored at approximately -70° C for biomarker analysis. No histopathologic or PCR analyses are performed on samples taken from these animals.

B. Laboratory Animals Required and Justification:

1. Non-animal Alternatives Considered: Non-animal alternatives are not feasible as the responses of interest require an integrated physiological system. The biomarkers being examined require interaction of the skin with the systemic circulation and with other organs such as the liver. Human skin, *ex vivo*, obtained through a local tissue network, may be used for limited studies to assess biomarkers which may arise from the skin itself following HD exposure. The human skin obtained from local tissue networks is not associated with any unique patient identifiers, hence the donor identity is completely anonymous.

2. Animal Model and Species Justification: Few laboratory animal species are known to microvesicate following HD exposure. One animal species which has been shown to microvesicate following percutaneous HD exposure is the euthymic hairless mouse (R. Casillas, Personal Communications, 1996). This model exhibits the pathology desired following HD exposure and allows for multiple exposure sites per animal.

3. Laboratory Animals:

   a. Genus & Species - *Mus musculus* (Euthymic Hairless Mouse)

   b. Strain/Stock - Crl:SKH1-hrBR

   c. Source/Vendor - Charles River Laboratories

   d. Age - Mice will be at least 21 days of age.

   e. Weight - Mice weighing 20 g at a minimum are used.

   f. Sex - Male

4. Total Number of Animals Required:

   a. Mice - 306

      (1) Dose Response Studies - 14 mice
      (2) TX Evaluation Studies - 60 mice
      (3) Endpoint Optimization -15 mice
      (4) Module I Studies - 36 mice
      (5) Module II Studies - 97 mice
      (6) Module III Studies - 84 mice

   b. Seventy extra mice are requested to cover technical problems and uncertainty about the nature of the dose range studies for HD vapor cap exposures. If additional animals are needed due to technical or unavoidable circumstances, or to expanded technical scope, Battelle's Institutional Animal Care and Use Committee (IACUC) procedures for requesting approval for additional animals will be followed.

5. Refinement, Reduction, Replacement:

   a. Refinement - If a suitable biomarker is identified, then it may be possible to reduce the amount of histopathologic analyses required for evaluations of candidate antivesicant compounds. A biomarker would provide a

quantitative measure as opposed to a semiquantitative measurement, thereby increasing the sensititivity of the evaluation processes.

b. Reduction - Steps have been taken to reduce animal use whenever possible. Studies are performed over a series of days which allows the use of a common control group which serves to minimize the number of animals required. Data from the client are being used to minimize the scope of the HD vapor duration-response studies. A main objective of these studies is to identify a quantifiable biomarker that can be used in place of, or in conjunction with, histopathologic evaluation for assessing candidate compound efficacy. The use of a quantitative endpoint should be a more sensitive endpoint than histopathology and should allow for a reduction in animal use in future testing.

c. Replacement - At this time, alternative procedures to provide an evaluation of an integrated organ system are not available.

C. Technical Methods:

1. Pain:

a. USDA (Form 18-3) Pain Category -

(1) No Pain - 70 mice

(2) Alleviated Pain - 306 mice

(3) Unalleviated Pain or Distress - 0 animals

b. Pain Alleviation -

(1) Anesthesia/Analgesia/Tranquilization - Animals are anesthetized with carbon dioxide or halothane prior to performing cardiac puncture procedures and are euthanatized while still anesthetized. During HD exposure periods when the animals are positioned, they are anesthetized using appropriate dosages of ketamine and xylazine.

Previous experience has indicated that pain or distress after HD exposure and decontamination does not exist. However, should evidence of pain or distress be noted during the study (e.g., decreased activity, anorexia, or self mutilation), pain will be alleviated with buprenorphine at an approximate dose of 2.5 mg/kg s.c. or i.p.

External stimuli and manipulation are minimized to decrease any associated anxiety.

(2) Paralytics - Not applicable

c. Alternatives to Painful Procedures -

(1) Source(s) Searched - TOXLINE

(2) Date of Search - 1/94 to 4/95

(3) Key Words of Search - sulfur mustard and pain; sulfur mustard and distress, sulfur mustard and discomfort, sulfur mustard and anxiety.

(4) Results of Search - No information using the key word combinations were found.

d. Painful Procedure Justification - No pain or discomfort to the animals is expected to result from cutaneous administration of either HD or the TXs used in this study.

2. Prolonged Restraint: Not applicable

3. Surgery: Not applicable

4. Animal Manipulations:

a. Injections - Mice are injected either i.p., s.c. or i.m. using a 25 or 27 g needle affixed to a 1-mL tuberculin syringe.

b. Biosamples -

(1) No biological samples will be taken prior to the time of sacrifice. Blood samples may be taken from anesthetized animals by cardiac puncture or from the retroorbital plexus and collected in serum tubes or placed into serum tubes after collection. Following centrifugation, the serum is stored at approximately -70° C until analyses.

(2) Skin samples for histopathologic evaluation are taken from euthanatized animals and placed in 10 percent neutral buffered formalin.

Skin samples also are taken from euthanatized animals for the purpose of measuring myeloperoxidase activity, and interleukin, and SAP levels. Skin samples are snap-frozen in liquid nitrogen and stored at approximately -70° C until used. The samples are processed by grinding in a liquid nitrogen-cooled mortar or, alternatively, tissue may be pulverized. The sample is reconstituted in a small volume of ice-cold buffer, transferred to a chilled test tube, and centrifuged in a prechilled centrifuge head at approximately 16,000 x g for 15 min at approximately 4° C. If a mortar and pestle is used for grinding the tissue, the mortar will be washed with a small volume of ice-cold buffer (such as 50 mM potassium phosphate buffer, pH 6.0, containing 0.5 percent hexadecyltrimethyl-ammonium bromide) and the wash material combined with the reconstituted sample contained in the test tube. After centrifugation, the supernatant may be used and/or aliquoted and stored at approximately -70° C.

Skin samples also are collected from euthanatized animals for performing PCR analyses of ornithine decarboxylase (ODC) or interleukin messenger ribonucleic acid (mRNA) levels. These samples will be snap-frozen in liquid nitrogen and stored at approximately -70° C. The tissues are transferred on dry ice to The Ohio State University (OSU) for analysis.

c. Animal Identification - Animals are identified by ear tagging, tail tattooing, or other appropriate measure. Animals may also be identified by cage cards where appropriate.

d. Behavioral Studies - Not applicable

e. Other Procedures -

(1) On the day of study, the animals are weighed to the nearest 0.5 gm using a calibrated balance. The exposure areas are delineated and a vapor cap mounting assembly is centered over the animal's back (four exposure sites per animal dorsum). Vapor cap mountings are prepared from double-sided carpet tape strips with holes punched through the assembly. The top protective surface of the carpet tape is not removed until after the templates are placed on the animal. The punched adhesive vapor cap mounting are centered on the animal's back to produce exposure sites on each side of the midline. Each exposure area is marked at the outermost edge of the hole with a permanent marker. Just prior to exposure, the protective tape assembly coverings are

removed with forceps to expose fresh adhesive surfaces to hold the vapor caps in place.

(2) HD Exposure - Following measurement of baseline skin erythema, the animals are anesthetized and placed into a chemical fume hood. The animals are positioned on a surface (e.g., surgical board or cardboard) in sternal recumbency for HD exposure. During anesthesia and HD exposure, animals may be placed on a warm-water perfused heating pad. Cutaneous exposure to HD vapor is done according to the methods described in SOP MREF II-009. Briefly, 10 μl of neat HD is pipetted onto a filter paper disk (Whatman #2) lodged in the inside top surface of a disposable plastic vial cap. The quantity of HD is sufficient to completely saturate the filter disc without run-off. The cap is inverted over the circular hole of the assembly and pressed onto the double sided tape. The time of vapor exposure for mice is determined from the outcome of the HD vapor duration-response studies. Forceps are used to apply or remove caps (ending exposures) and to remove tape assemblies from the skin. After exposure, animals are housed in polycarbonate cages in a fume hood until they are euthanatized. The exposed areas of the animals are not decontaminated.

5. Adjuvant: Not applicable

6. Study Endpoints:

a. Ornithine Decarboxylase (ODC) - Tissue samples are snap frozen in liquid nitrogen, and stored at approximately -70° C. Samples are submitted to OSU for ribonucleic acid isolation and reverse transcriptase polymerase chain reaction of ODC message.

b. Myeloperoxidase (MPX) - MPX is measured using the spectrophotometric method of Bradley et al., (1982) as adapted to allow the use of a microtiter plate reader (Bongiavanni et al., 1993) or COBAS Fara centrifugal analyzer. These data will be standardized on a total protein basis.

c. Interleukin - IL-1 alpha and IL-6 are measured using commercially available enzyme immunoassay kits. These data are standardized on a protein basis. Levels of mRNA for these cytokines may be examined using a reverse transcription PCR (RT-PCR) technique.

d. Granuloyte Macrophage - Colony Stimulating Factor (GM CSF) is measured using RT-PCR.

e. Acute Phase Reactive Protein - An antibody to serum amyloid P (SAP), the major acute phase reactive protein in mice, is available commercially. Under Pre-Task Pilot Study 94-20, another acute phase reactive protein, haptoglobin, was shown to be elevated in swine skin exposed to HD. An enzyme immunoassay is used to evaluate SAP levels. These data are standardized on a protein basis.

f. Protein - Protein concentration in supernatants will be determined using the Pierce Coomassie Protein Assay Reagent with bovine serum albumin as a standard.

g. Edema - Edema measurements are made by comparing the wet weight of HD-exposed skin punch biopsies to the wet weight of unexposed control skin punch biopsies. Replicate sites are averaged on each animal, and a single skin weight thickness change is calculated for control and exposure sites.

h. Erythema - Erythema (redness) is determined by light reflectance using a Minolta Chroma Meter (Braue et al., 1993). Four replicate readings will be taken prior to exposure and at the indicated times following exposure. If replicate sites exist on an animal, the replicates are averaged and a single a* difference value is calculated for control and exposure sites.

i. Histopathologic Evaluations - Approximately 5 μm sections are taken through the center of the lesion of paraffin-embedded specimens, mounted on slides, and stained with hematoxylin and eosin. The specimens are assessed for the presence of microblisters, epidermal necrosis, follicular necrosis, pustular epidermitis, and intracellular edema. Histopathologic endpoints are scored on a "0" to " 4" semi-quantitative scale where a "1" indicates that the pathologic change is negligible and only occurs in one or two discrete foci and "4" denotes maximum severity and diffusion. The intermediate scores, "2" and "3", span the intermediate area and take into account both amount and distribution of damage. The histopathological markers have the following definitions:

- **Intracellular edema** (ballooning degeneration, hydropic degeneration, vacuolar degeneration) of the epidermis is characterized by increased cell size, cytoplasmic pallor, and displacement of the nucleus to the periphery of affected cells; refers to all layers of the epidermis.

- **Epidermal necrosis** primarily refers to the nuclear morphology of those cells in the epidermis and includes condensation and shrinkage

(pyknosis), fragmentation (karyorrhexis), and dissolution (karyolysis) of the nucleus. Basal cells are the cells most affected by HD.

- **Pustular epidermitis** is characterized by the presence of neutrophils within the epidermal layer. Under normal conditions and without the appropriate stimuli (inflammatory mediator release), there should be no neutrophils present.

- **Microblisters** are defined as a visible (light microscope level) separation and loss of attachment of the basal cell layer from the underlying basement membrane. Must represent the loss, or dissolution, of at least two adjacent basal cells. Frequently within this newly created space there will be cellular debris, neutrophils, and macrophages (i.e., a micropustule).

- **Follicular necrosis** refers to the destruction of the basal cell layer and other epidermal layers which invaginate into the dermis and line the hair follicle.

7. Euthanasia: Mice will be sacrificed by halothane overdose, carbon dioxide overdose, or other method approved by the American Veterinary Medical Association's Panel on Euthanasia.

D. Veterinary Care:

1. Husbandry Considerations: Battelle's Animal Resources Facilities have been registered with the U.S. Department of Agriculture (USDA) as a Research Facility (Number 31-R-21) since August 14, 1967, and are periodically inspected in accordance with the provisions of the Federal Animal Welfare Act. Animals for use in research are obtained only from laboratory animal suppliers duly licensed by the USDA. Battelle's statement of assurance regarding the Department of Health and Human Services (DHHS) policy on humane care of laboratory animals was accepted by the Office of Protection from Research Risks, National Institutes of Health (NIH) on August 27, 1973. Animals at Battelle are cared for in accordance with the guidelines set forth in the "Guide for the Care and Use of Laboratory Animals" (NIH Publication No. 86-23) and/or in the regulations and standards as promulgated by the Agricultural Research Service, USDA, pursuant to the Laboratory Animal Welfare Act of August 24, 1966 as amended.

   Accreditation - On January 31, 1978, Battelle's Columbus Operation received full accreditation of its animal-care program and facilities from the American

Association for Accreditation of Laboratory Animal Care (AAALAC). Battelle's full accreditation status has been renewed after every inspection since the original accreditation. The MREF is a part of the facilities granted full accreditation.

a. Mice - Animals will be group housed or housed individually in polycarbonate cages. Following HD exposure, mice will be housed in a chemical fume hood for the indicated holding period. Animals will have access to food and water, *ad libitum*, except on the morning of exposure. While housed in the fume hood system, the cages may be situated on a warm water-perfused heating pad, and the fume hood sashes are kept closed during the overnight holding period to prevent drafts.

b. Animals are quarantined for a minimum of five days.

c. Fluorescent lighting is used with a 12-hr light cycle per day, except when animals are maintained for the approximately 24-hr period following exposure in the chemical fume hood.

d. Temperature - Maintained at 64-79° F. At least 95 percent of the twice daily readings will be within this range.

e. Relative Humidity - Maintained at 40-70 percent. At least 95 percent of the twice daily readings will be within this range.

f. Diet - Purina Rodent Chow is available *ad libitum* except immediately prior to study. No contaminants which would affect the results of the study are known to be present in the feed.

g. Water - Water is supplied from the Battelle West Jefferson water system and available *ad libitum* except immediately prior to study. No contaminants which would affect the results of the study are known to be present in the water.

h. Special Husbandry Considerations - Not applicable

2. Attending Veterinary Care:

a. Animals are examined upon receipt.

b. Animals are examined at least once a day by trained technical personnel. If any problems are observed, a staff veterinarian is notified.

c. On the day of exposure and periodically throughout the post-exposure time period, animals are observed by trained personnel. If an animal becomes moribund, it will be euthanatized.

3. Enrichment Strategy: Polyvinyl chloride tubing (approximate internal diameter of four inches) cut into half lengthwise is placed in cages with the mice.

E. Data Analysis: For HD vapor duration-response studies, the histopathology is analyzed using linear or nonlinear (e.g., logistic or probit regression) models fitted to the data to determine the HD vapor duration-response relationship for each endpoint. The fitted models are used to calculate the HD vapor durations corresponding to specified percentiles of the duration-response relationships. If adequate fits are not achieved via the regression or nonlinear regression models, then nonparametric procedures may be used. The difference between pre and post HD exposure erythema measurements, based on the reflectance color meter, are calculated and statistically analyzed using an analysis of variance (ANOVA) model. The assumption of approximate normality for the distribution of erythema data is assessed visually. If this assumption is grossly violated then either 1) a transformation may be applied to the data prior to carrying out the tests, or 2) the analysis may be conducted using nonparametric or categorical methods. Similar analysis are conducted for the edema data.

For candidate pre- and post- exposure treatment compound evaluations, erythema and edema results at sham, HD-exposed, TX, and HD-exposed/TX sites will be statistically compared for each of the four TX compounds using a repeated measures ANOVA model. In addition, results of the four TX compounds will be statistically compared. If the assumption of approximate normality for the distribution of erythema and/or edema data is grossly violated then either 1) a transformation may be applied to the data prior to carrying out the tests, or 2) the analysis may be conducted using nonparametric or categorical methods. For histologic endpoints, McNemar's test will be used to compare incidence of histologic endpoints among the four sites. Logistic regression, probit analyses, or categorical analyses may be used to compare histologic endpoints among the four TX compounds. Transition of the model will be considered successful if the incidence of microvesication observed in the animals exposed at Battelle is qualitatively similar to results at USAMRICD.

For Module I evaluations, biological responses measured in HD-exposed animals will be compared to those of naive animals to determine if biomarker responses in serum and tissue samples are statistically different for the two groups of animals. Method of statistical comparison (logistic regression, probit analysis, categorical analysis, or ANOVA) will depend on the measured response.

For Module II evaluations, erythema, edema and biological responses measured at HD-exposed/TX sites will be compared to those at HD-exposed sites to determine if results are statistically different for sites administered TX and, sites without treatment for each candidate compound. In addition, results of the four TX compounds and the HD exposed only group will be statistically compared using methods employed in candidate pre- and post- treatment compound and Module I evaluations.

For Module III evaluations, biological responses measured in serum samples of treated/HD-exposed animals will be statistically compared to those of naive and HD-exposed animals to determine if biomarker responses are statistically different among the different groups of animals. Statistical methods employed in Module I evaluations may be used for these comparisons.

F. Investigator and Technician Qualifications/Training: All technical staff members involved in the receipt, care, and use of animals are AALAS certified laboratory animal technicians or in training for certification. All training documentation is maintained in personnel training files. Dr. James Blank has four years of experience in the handling and use of rodent species for experimental purposes. Dr. Ronald Menton is a biostatistician with over seven years of experience in designing biostudies and applying statistical design to minimize and reduce animal usage. Dr. Tracy Peace has a veterinary medicine degree and is a diplomate of the American College of Laboratory Animal Medicine.

VI. Biohazard/Safety: The chemicals and hazardous wastes used or generated in this protocol will be handled in accordance with all applicable state and federal guidelines, regulations and Battelle standard operating procedures to ensure that no significant adverse environmental effects occur. All HD Chemical Surety Material (CSM) work will be conducted in accordance with all applicable Facility Safety and Surety Plan (FSSP) and Standard Operating Procedures (SOPs). Standard monitoring procedures are in place to assure safety in conjunction with the use of HD.

VII. Assurances: As Primary Investigator on this protocol I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a deviation is specifically approved by Battelle's IACUC and the COR.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used".

D. Biohazard/Safety: I have taken into consideration, and I have made the proper coordinations regarding all applicable rules and regulations regarding radiation protection, biosafety, recombinant issues, etc., in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures/manipulations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain, or distress will be caused as a result of the procedures/manipulations.

F. Responsibility: I acknowledge the inherent moral and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" which the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible, and conducting humane and lawful research.

James A. Blank, Ph.D., Study Director

G. Painful Procedures: I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals that will be relieved with the use of anesthetics, analgesics, and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of the proposed experiment.

James A. Blank, Ph.D., Study Director

VIII. Enclosures:

A. Literature Searches

IX. References:

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Evaluation of Biomarkers for Sulfur Mustard Exposure in the
Euthymic Hairless Mouse Model

Protocol Amendment No. 1

Change No. 1: Page 7, Section V.A.4.

Change from:

4. Module I Evaluations: Biological Endpoint Screening: Endpoints are screened for potential utility by performing evaluations at an early (~2 to 4 hr) and later (~24 hr) time following exposure to an HD vapor causing a high incidence of microvesication. Histopathologic measurements are not made in these evaluations. Biomarker responses in serum and tissue samples may be evaluated using four groups of animals as shown in the following table:

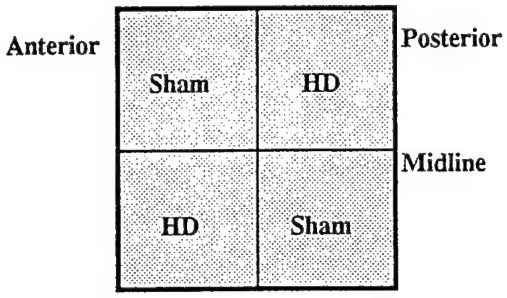
Group	Group Description	N[A]	Sample Time/Source 4 HR	24 HR
A	Naive	8	Plasma & Skin	
B	HD-Exposed	8	Plasma & Skin	
C	Naive	8		Plasma & Skin
D	HD-Exposed	8		Plasma & Skin

[A] Number of animals per group.

a. HD exposures are performed over a two day period with half the animals of each experimental treatment group being exposed on each of two dosing days.

b. Dosing grids are delineated on the dorsa of the animals and the vapor cap assemblies are put in place. Baseline erythema measurements are taken. Animals are anesthetized with ketamine and xylazine, and positioned on a surface (e.g., surgical board, cardboard, etc).

c. Animals from Groups A and C receive four sham HD exposures per animal, while animals in Groups B and D are exposed to HD vapor as indicated in the following schematic.



d. At the indicated times post exposure, erythema is evaluated. The animals are then anesthetized and blood specimens are collected by cardiac puncture and placed into serum tubes. Following euthanasia, tissue samples are taken and edema measurements performed as described in a later section of this protocol. The tissue specimens are snap-frozen in liquid nitrogen and stored with the serum specimens at approximately - 70° C for biomarker analysis. No histopathology is performed on the skin specimens.

e If HD does not cause significant ($p < 0.05$) alteration of an endpoint relative to control samples, then the endpoint is not tested further. If HD does cause a significant ($p < 0.05$) response alteration, then the endpoint may be evaluated through Modules II and/or III as directed by the COR.

Change to:

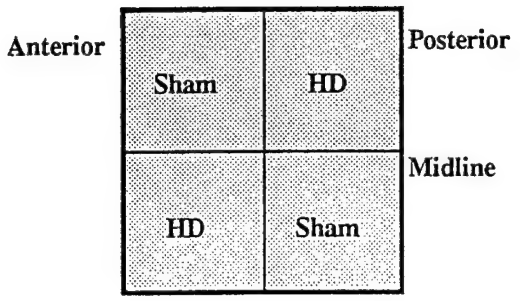
4. Module I Evaluations: Biological Endpoint Screening: Endpoints are screened for potential utility by performing evaluations at an early (~1 to 2 hr), intermediate (~ 4 to 6 hr), and late (~24 hr) time following exposure to an HD vapor causing a high incidence of microvesication. Histopathologic measurements are not made in these evaluations, although immunohistochemistry may be performed on select endpoints. Biomarker responses in serum and tissue samples may be evaluated using the groups of animals shown in the following table:

Group	Group Description	N[a]	Sample Time/Source		
			1 HR	4 HR	24 HR
A	Naive	8	Plasma & Skin		
B	HD-Exposed	8	Plasma & Skin		
C	Naive	8		Plasma & Skin	
D	HD-Exposed	8		Plasma & Skin	
E	Naive	8			Plasma & Skin
F	HD-Exposed	8			Plasma & Skin
G	HD-Exposed	4	Skin[b]		
H	HD-Exposed	4		Skin[b]	
I	HD-Exposed	4			Skin[b]
J	HD-Exposed	4	IHC[c]		
K	HD-Exposed	4		IHC[c]	
L	HD-Exposed	4			IHC[c]

[a] Number of animals per group.
[b] Skin specimens will be used for ribonuclease protection assay (RPA)
[c] Immunohistochemistry

a. Dosing grids are delineated on the dorsa of the animals and vapor cap assemblies put in place. Animals are anesthetized with ketamine and xylazine, and positioned on a surface (e.g., surgical board, cardboard, etc).

b. Animals from Groups A, C, and E receive four sham HD exposures per animal, while animals in Groups B, D, F, G, H, I, J, K, and L are exposed to HD vapor as indicated in the following schematic.



c. At the indicated times post exposure, the animals are anesthetized and blood specimens are collected by cardiac puncture and placed into serum tubes. Following euthanasia, tissue samples are taken and edema measurements performed. Blood specimens and edema measurements will not be taken on animals from Groups G through L. Except for specimens from Groups J, K, and L, the tissue specimens are snap-frozen in liquid nitrogen and stored with the serum specimens at approximately - 70° C for biomarker analysis. Specimens from Groups J, K, and L will be fixed in formalin, then transferred to an isotonic solution such as phosphate buffered solution. These specimens are evaluated using immunohistochemistry by USAMRICD.

d. HD does not cause significant ($p < 0.05$) alteration of an endpoint relative to control samples, then the endpoint is not tested further. If HD does cause a significant ($p < 0.05$) response alteration, then the endpoint may be evaluated through Modules II and/or III as directed by the COR

Reasons for Change: A third time point has been included in these analyses to evaluate biomarker response at an intermediate time following exposure. Data from other studies indicate that a 1 to 2 hr time point and a 4 to 6 hr time point may be optimal for measuring some of the endpoints [personal communications with CPT Robert Casillas]. Erythema measures will not be taken. Initial observations made in this task indicate that these measures using the Minolta Chromameter are difficult with animals of this size, and skin blanching from animal restraint may introduce artifact. Tissue specimens also will be taken for RPA analyses of biomarkers. This can be performed using RPA panels, which consist of multiple endpoint analyses with each tissue specimen.

Impact on Study: These changes should increase the likelihood of identifying a biomarker for HD-induced injury, and should enhance the understanding of HD-induced inflammation and pathology.

Change No. 2: Page 8, Section V.A.5.

Change from:

5. Module II Evaluations: Dermal Biomarker Qualification: The biomarkers and time assessment parameters for Module II evaluations are determined from Module I results. If an HD-induced alteration is observed only at 24 hr, then the 4 hr evaluations are not performed. If an HD-induced alteration is observed only at 4 hr, then the 24 hr time point is not evaluated. If the endpoints are altered at both time points, then Module II assessments may be made at both times, as directed by the COR.

   TX compounds that have been shown to be effective in the hairless mouse model are used for Module II evaluations. Up to four TX compounds may be evaluated with skin preparations being analyzed for biomarkers.

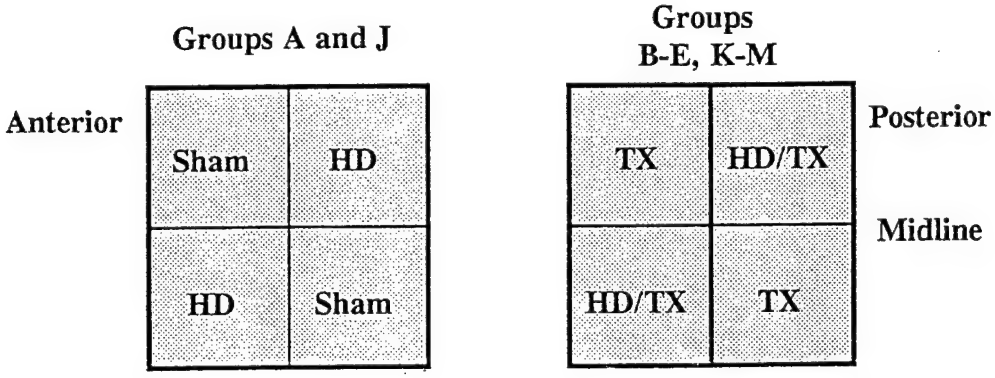
Group	Group Description	N	4 Hr Measurements	24 Hr Measurements
A	HD	8	Erythema, Edema, Biomarker	
B	HD/TX#1	8	Erythema, Edema, Biomarker	
C	HD/TX#2	8	Erythema, Edema, Biomarker	
D	HD/TX#3	8	Erythema, Edema, Biomarker	
E	HD/TX#4	8	Erythema, Edema, Biomarker	
F	HD/TX#1	3	Erythema, Edema, PCR[A]	
G	HD/TX#2	3	Erythema, Edema, PCR	
H	HD/TX#3	3	Erythema, Edema, PCR	
I	HD/TX#4	3	Erythema, Edema, PCR	
J	HD	8		Erythema, Edema, Biomarker
K	HD/TX#1	8		Erythema, Edema, Biomarker
L	HD/TX#2	8		Erythema, Edema, Biomarker
M	HD/TX#3	8		Erythema, Edema, Biomarker
N	HD/TX#4	8		Erythema, Edema, Biomarker

[A] Polymerase chain reaction.

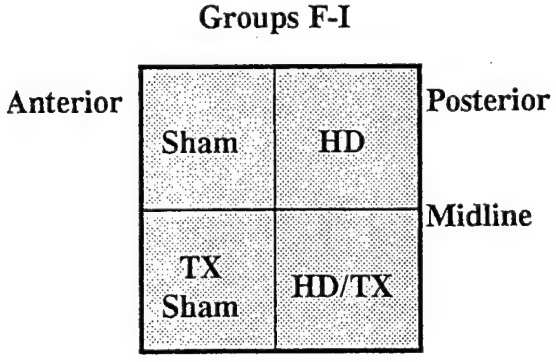
a. HD exposures for Groups A to I are performed over a four day period with animals from each group being exposed on each dosing day. HD exposures for Groups J to N are performed over three days with animals from each group being exposed on each dosing day.

b. Dosing grids are delineated on the dorsa of the animals and the vapor cap assemblies are put in place. Baseline erythema measurements are taken. Animals are anesthetized with ketamine and xylazine, and positioned on a surface (e.g., surgical board, cardboard, etc).

c. The dosing schematic for the various groups are presented in the following illustrations:



The following dosing schematic is used on the groups exposed to collect skin specimens for PCR analyses.



d. At the indicated times post exposure, erythema is evaluated. The animals are euthanatized, tissue samples are taken, and edema measurements performed. The tissue specimens are snap-frozen in liquid nitrogen and stored at approximately -70° C for biomarker analysis. No histopathology is performed in this module.

Change to:

5. Module II Evaluations: Dermal Biomarker Qualification: The biomarkers and time assessment parameters for Module II evaluations are determined from Module I results. If an HD-induced alteration is observed only at 24 hr, then the early and intermediate time point evaluations are not performed. If an HD-induced alteration is observed only at the early time point, then the intermediate and 24 hr time point are not evaluated. Likewise for changes observed at the intermediate time point, evaluations will not be performed at the early and late time points. If the endpoints are altered at all three time points, then Module II assessments may be made at times directed by the COR.

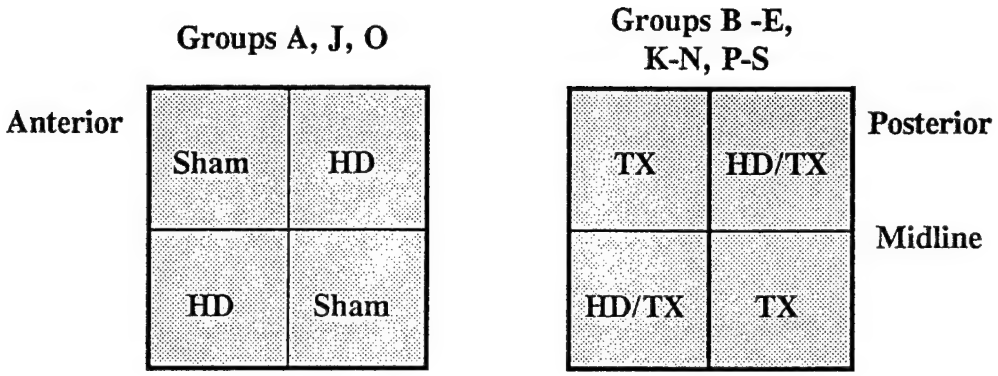
   TX compounds that have been shown to be effective in the hairless mouse model are used for Module II evaluations. Up to four TX compounds may be evaluated with skin preparations being analyzed for biomarkers.

Group	Group Description	N	1-2 Hr Measurements	4-6 Hr Measurements	24 Hr Measurements
A	HD	8	Edema, Biomarker		
B	HD/TX#1	8	Edema, Biomarker		
C	HD/TX#2	8	Edema, Biomarker		
D	HD/TX#3	8	Edema, Biomarker		
E	HD/TX#4	8	Edema, Biomarker		
F	HD/TX#1	3	RPA		
G	HD/TX#2	3	RPA		
H	HD/TX#3	3	RPA		
I	HD/TX#4	3	RPA		
J	HD	8		Edema, Biomarker	
K	HD/TX#1	8		Edema, Biomarker	
L	HD/TX#2	8		Edema, Biomarker	
M	HD/TX#3	8		Edema, Biomarker	
N	HD/Tx#4	8		Edema, Biomarker	
O	HD	8			Edema, Biomarker
P	HD/TX#1	8			Edema, Biomarker
Q	HD/TX#2	8			Edema, Biomarker
R	HD/TX#3	8			Edema, Biomarker
S	HD/Tx#4	8			Edema, Biomarker

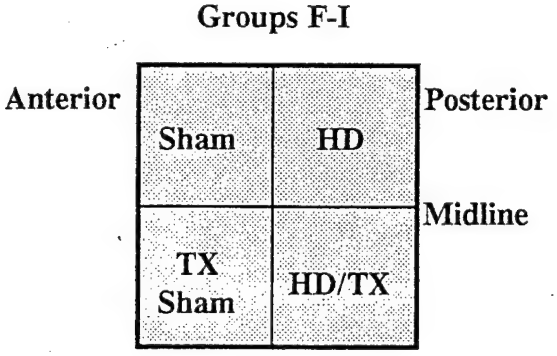
a. HD exposures for various groups are performed over multiple days.

b. Dosing grids are delineated on the dorsa of the animals and the vapor cap assemblies are put in place. Animals are anesthetized with ketamine and xylazine, and positioned on a surface (e.g., surgical board, cardboard, etc).

c. The dosing schematic for the various groups are presented in the following illustrations:



The following dosing schematic is used on the groups exposed to collect skin specimens for RPA analyses.



d. At the indicated times post exposure, the animals are euthanatized and tissue samples are taken. Edema measurements will be performed on all groups except those involving RPA analyses. The tissue specimens are snap-frozen in liquid nitrogen and stored at approximately -70° C for biomarker analysis. No histopathology is performed in this module.

Reason for Change: A third time point has been included for biomarker evaluations. Analyses to be performed by RT-PCR will be performed by RPA analyses.

Impact on Study: This change should increase the likelihood of identifying a biomarker for HD exposure.

Change No. 3: Page 10, Section V.A.6. Erythema measurements will not be performed.

Reason for Change: Initial observations made in this task indicate that these measures, which use the Minolta Chromameter, are difficult with animals the size of the mouse, and skin blanching from animal restraint induces artifact.

Change No. 4: Page 12, Section V.B.4.

Change from:

4. Total Number of Animals Required:

   a. Mice - 306

      (1) Dose Response Studies - 14 mice
      (2) TX Evaluation Studies - 60 mice
      (3) Endpoint Optimization -15 mice
      (4) Module I Studies - 36 mice
      (5) Module II Studies - 97 mice
      (6) Module III Studies - 84 mice

   b. Seventy extra mice are requested to cover technical problems and uncertainty about the nature of the dose range studies for HD vapor cap exposures. If additional animals are needed due to technical or unavoidable circumstances, or to expanded technical scope, Battelle's Institutional Animal Care and Use Committee (IACUC) procedures for requesting approval for additional animals will be followed

Change to:

4. Total Number of Animals Required:

   a. Mice - 306

      (1) Dose Response Studies - 14 mice
      (2) TX Evaluation Studies - 60 mice
      (3) Endpoint Optimization -15 mice

(4) Module I Studies - 76 mice
(5) Module II Studies - 137 mice
(6) Module III Studies - 84 mice

b. Ninety additional mice are requested because of the uncertainty of the dose ranges for HD vapor cap exposures and the additional of evaluations at additional time points. Battelle's Institutional Animal Care and Use Committee (IACUC) procedures for requesting approval for additional animals will be followed

Reason for Change: Addition of a third time point, IHC measurements, and RPA measurements in Module I studies, and inclusion of a third time point in Module II evaluations have resulted in a need for additional animals.

Impact on Study: Additional information on the effect of HD on skin should be gained.

Change No. 5: Page 13, Section V.C.1.

Change from:

C. Technical Methods:

1. Pain:

a. USDA (Form 18-3) Pain Category -

(1) No Pain - 70 mice

(2) Alleviated Pain - 306 mice

(3) Unalleviated Pain or Distress - 0 animals

Change to:

C. Technical Methods:

1. Pain:

   a. USDA (Form 18-3) Pain Category -

      (1) No Pain - 90 mice

      (2) Alleviated Pain - 386 mice

      (3) Unalleviated Pain or Distress - 0 animals

Reason for Change: The scope of work has been expanded which increases the number of animals required for the study.

Impact on Study: Additional information on HD-induced skin toxicity should be gained.

Change No. 6: Page 16, Section V.C.6.

Change from:

6. Study Endpoints:

   a. Ornithine Decarboxylase (ODC) - Tissue samples are snap frozen in liquid nitrogen, and stored at approximately -70° C. Samples are submitted to OSU for ribonucleic acid isolation and reverse transcriptase polymerase chain reaction of ODC message.

   b. Myeloperoxidase (MPX) - MPX is measured using the spectrophotometric method of Bradley et al., (1982) as adapted to allow the use of a microtiter plate reader (Bongiavanni et al., 1993) or COBAS Fara centrifugal analyzer. These data will be standardized on a total protein basis.

   c. Interleukin - IL-1 alpha and IL-6 are measured using commercially available enzyme immunoassay kits. These data are standardized on a protein basis. Levels of mRNA for these cytokines may be examined using a reverse transcriptase PCR (RT-PCR) technique.

d. Granuloyte Macrophage - Colony Stimulating Factor (GM CSF) is measured using RT-PCR.

e. Acute Phase Reactive Protein - An antibody to serum amyloid P (SAP), the major acute phase reactive protein in mice, is available commercially. Under Pre-Task Pilot Study 94-20, another acute phase reactive protein, haptoglobin, was shown to be elevated in swine skin exposed to HD. An enzyme immunoassay is used to evaluate SAP levels. These data are standardized on a protein basis.

f. Protein - Protein concentration in supernatants will be determined using the Pierce Coomassie Protein Assay Reagent with bovine serum albumin as a standard.

g. Edema - Edema measurements are made by comparing the wet weight of HD-exposed skin punch biopsies to the wet weight of unexposed control skin punch biopsies. Replicate sites are averaged on each animal, and a single skin weight thickness change is calculated for control and exposure sites.

h. Erythema - Erythema (redness) is determined by light reflectance using a Minolta Chroma Meter (Braue et al., 1993). Four replicate readings will be taken prior to exposure and at the indicated times following exposure. If replicate sites exist on an animal, the replicates are averaged and a single a* difference value is calculated for control and exposure sites.

Change to:

6. Study Endpoints:

a. RPA will be used to evaluate RNA levels of a number of pro-inflammatory mediators. Biomarkers such as ornithine decarboxylase (ODC) and tenascin may also be measured. Specimens will be snap frozen in liquid nitrogen, and stored at approximately -70° C.

b. IHC will be performed on control and HD-exposed tissue specimens to evaluate the presence of a number of pro-inflammatory mediators. Tissue specimens will be fixed in neutral buffered formalin for approximately 4 hr,then transferred to an

isotonic solution such as phosphate buffered saline. Other methods of tissue fixation may be used as directed by the COR.

c. *In situ* hybridization may be performed on tissue specimens to evaluate the source of pro-inflammatory mediators.

d. Myeloperoxidase (MPX) - MPX is measured using the spectrophotometric method of Bradley et al., (1982) as adapted to the use of a microtiter plate reader (Bongiavanni et al., 1993) or COBAS Fara centrifugal analyzer. These data will be standardized on a total protein basis.

e. Interleukin - Proinflammatory mediators such as IL-1 alpha and IL-6 are measured using commercially available enzyme immunoassay kits. These data are standardized on a protein basis.

f. Acute Phase Reactive Protein - An antibody to serum amyloid P (SAP), the major acute phase reactive protein in mice, is available commercially. Under Pre-Task Pilot Study 94-20, another acute phase reactive protein, haptoglobin, was shown to be elevated in swine skin exposed to HD. An enzyme immunoassay is used to evaluate SAP levels. These data are standardized on a protein basis.

g. Protein - Protein concentration in supernatants will be determined using a standard protein assay such as the Pierce Coomassie Protein Assay Reagent.

h. Edema - Edema measurements are made by comparing the wet weight of HD-exposed skin punch biopsies to the wet weight of unexposed control skin punch biopsies. Replicate sites are averaged on each animal, and a single skin weight thickness change is calculated for control and exposure sites.

Reason for Change: RPA analyses will be performed instead of RT-PCR analyses. Erythema is no longer used as an endpoint.

Impact on Study: Changes should increase the information gained in this study.

Approvals:

James A. Blank, Ph.D., Study Director — Date: 20-JAN-98

LTC Richard R. Stotts, D.V.M., Ph.D.
USAMRICD — Date: 27 JAN 98

Reviewed and Registered by:

Elisha N. Morrison, M.S.
Quality Assurance Specialist — Date: 1/29/98
g

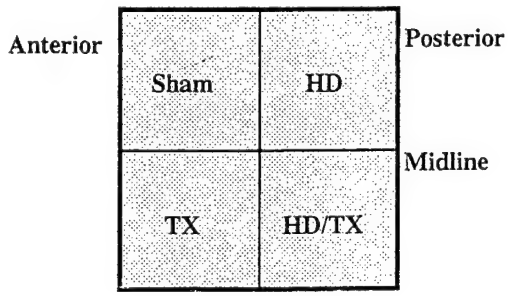
Evaluation of Biomarkers for Sulfur Mustard Exposure in the
Euthymic Hairless Mouse Model

Protocol Amendment No. 2

Change No. 1: Page 5, Section V.A.2.a. and V.A.2.b.

Change from:

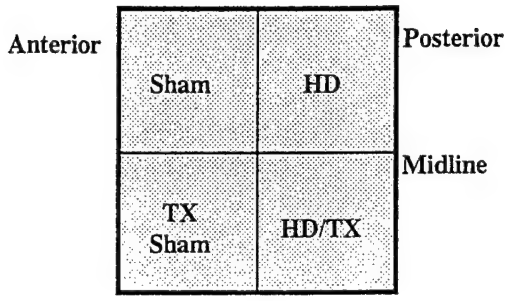
a. These evaluation studies will utilize the fixed HD vapor durations that had been shown previously to produce a high incidence of microvesication. Ten animals per TX are evaluated for changes in erythema, edema, and histopathology at approximately 24 hr following HD exposure. TX and HD exposures are applied to the animals as indicated in the following schematic:



b. The dosing sites are randomized from animal to animal to minimize experimental bias due to positional effects. All four TXs are evaluated concurrently, with animals from each group being exposed on each of three days. Erythema, edema, and histopathology measurements are made for each dose site.

Change to:

a. These evaluation studies will utilize the fixed HD vapor durations that had been shown previously to produce a high incidence of microvesication. Ten animals per TX are evaluated for changes in erythema, edema, and histopathology at approximately 24 hr following HD exposure. TX and HD exposures are applied to the animals as indicated in the following schematic:



b. The dosing sites are randomized from anteriorly to posteriorly for each animal to minimize experimental bias due to positional effects. Both the control and TX shams are always kept at the two anterior exposure sites or at the two posterior sites. All four TXs are evaluated concurrently, with animals from each group being exposed over a couple of days. Edema and histopathology measurements are made for each dose site.

Reason for Change: Discussions with the technical point of contact, CPT Robert Casillas, indicated that the dosing schematic would allow all treatment combinations to be applied to each animal.

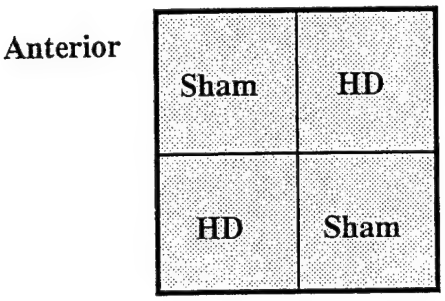
Impact of Change: This change may enhance the quality of data produced.

Change No. 2: Page 33, Section V.A.5.c.

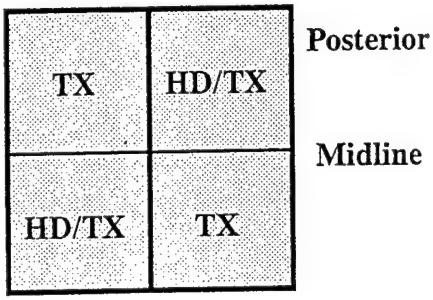
Change From:

c. The dosing schematic for the various groups are presented in the following illustrations:

Groups A, J, O

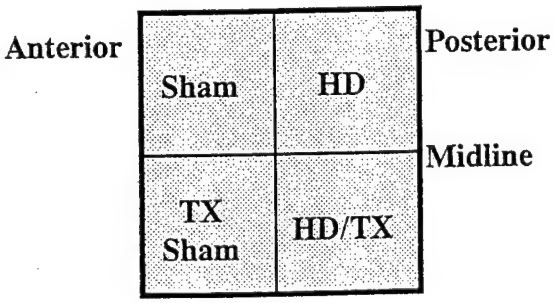


Groups B -E, K-N, P-S



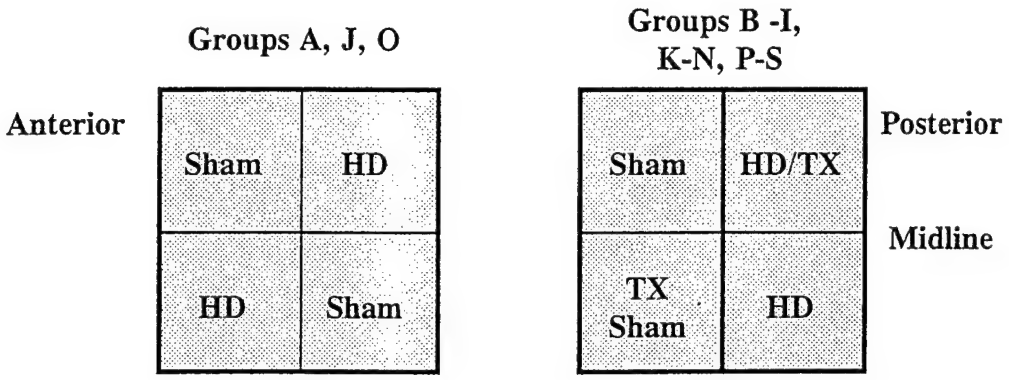
The following dosing schematic is used on the groups exposed to collect skin specimens for RPA analyses.

Groups F-I



Change To:

c. The dosing schematic for the various groups are presented in the following illustrations:



The dosing sites are randomized from anteriorly to posteriorly for each animal to minimize experimental bias due to positional effects. Both the control and TX shams are always kept at the two anterior exposure sites or at the two posterior sites. All four TXs are evaluated concurrently, with animals from each group being exposed over a couple of days. Edema and histopathology measurements are made for each dose site.

Reason for Change: Discussions with the technical point of contact, CPT Robert Casillas, indicated that the randomization scheme shown would account for all treatment combinations in each animal.

Impact of Change: This change may enhance data quality.

Approvals:

James A. Blank, Ph.D., Study Director — Date: 26-MAR-98

LTC Richard R. Stotts, D.V.M., Ph.D.
USAMRICD — Date: 30 MAR 98

Reviewed and Registered by:

Elisha N. Morrison, M.S.
Quality Assurance Specialist — Date: 3/31/98

Evaluation of Biomarkers for Sulfur Mustard Exposure in the Euthymic Hairless Mouse Model

Protocol Amendment No. 3

Change No. 1: Page 10, Section V.6.

Change from:

6. Module III Evaluations: Serum Biomarker Qualification: If a biomarker in the serum samples was altered significantly ($p < 0.05$) within 4 hr of HD exposure in Module I evaluations, then the biomarker may be examined in naive animals, treated animals, HD-exposed animals, and HD-exposed animals that have been given a TX. These evaluations are performed only on blood samples collected at the early (~4 hr) time point unless otherwise directed by the COR.

GROUP	Group Description	N	4 Hr Measurements
A	Naive	8	Erythema, Edema, plasma biomarker
B	HD	8	Erythema, Edema, plasma biomarker
C	TX#1	8	Erythema, Edema, plasma biomarker
D	HD+TX#1	8	Erythema, Edema, plasma biomarker
E	TX#2	8	Erythema, Edema, plasma biomarker
F	HD+TX#2	8	Erythema, Edema, plasma biomarker
G	TX#3	8	Erythema, Edema, plasma biomarker
H	HD+TX#3	8	Erythema, Edema, plasma biomarker
I	TX#4	8	Erythema, Edema, plasma biomarker
J	HD+TX#4	8	Erythema, Edema, plasma biomarker

a. Each experimental group consists of eight animals. The exposures may be performed over an eight day period with an animal per treatment group being exposed each day.

b. Dosing grids are delineated on the dorsa of the animals and the vapor cap assemblies are put in place. Baseline erythema measurements are taken.

Animals are anesthetized with ketamine and xylazine, and positioned on a surface (e.g., surgical board, cardboard, etc).

c. The dosing schematics for the various groups are presented in the following illustrations:

Change to:

6. Module III Evaluations: Mouse Ear Biomarker Analyses: Investigators at USAMRICD, under a separate protocol, will collect ear specimens from CD1 mice which have been pretreated with therapeutic compound and/or exposed to HD. Tissue specimens which have been snap-frozen in liquid nitrogen will be sent to the MREF for biomarker analyses. The therapeutic compounds used at USAMRICD are the same as used under Module II testing of this protocol. The endpoints performed on the submitted tissue specimens will be similar to those performed under Module II.

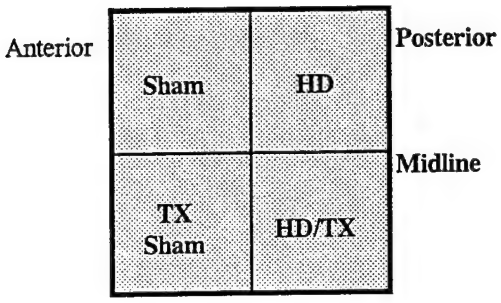
Reason for Change: This change will provide a comparison of the inflammatory characteristics between the CD1 mouse model which uses a percutaneous liquid HD challenge on the ear with the euthymic hairless mouse which is exposed percutaneously on the back to HD vapor.

Impact of Change: The comparison between the CD1 and euthymic hairless mice should further our understanding of the inflammation process induced by liquid and vaporous HD exposures. In addition, since the CD1 mouse ear swelling model is currently used under MREF Protocol 116 for evaluating candidate therapeutic compounds, these efforts should provide a transition for the potential use of these biomarkers for candidate compound evaluations.

Change No. 2: Page 40, Section V.A.2.a. and V.A.2.b.

Change from:

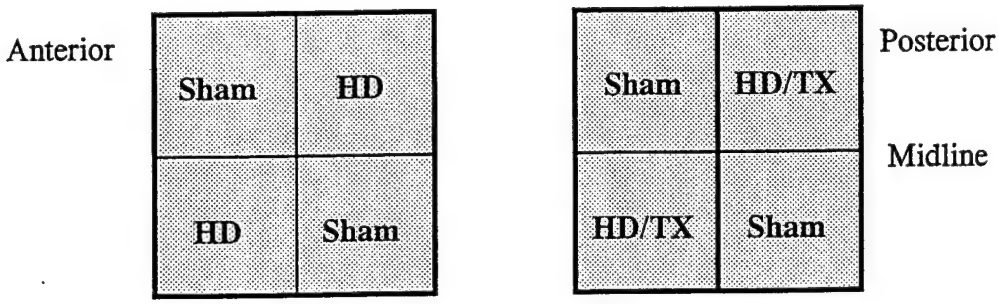
a. These evaluation studies will utilize the fixed HD vapor durations that had been shown previously to produce a high incidence of microvesication. Ten animals per TX are evaluated for changes in erythema, edema, and histopathology at approximately 24 hr following HD exposure. TX and HD exposures are applied to the animals as indicated in the following schematic:



b. The dosing sites are randomized from anteriorly to posteriorly for each animal to minimize experimental bias due to positional effects. Both the control and TX shams are always kept at the two anterior exposure sites or at the two posterior sites. All four TXs are evaluated concurrently, with animals from each group being exposed over a couple of days. Edema and histopathology measurements are made for each dose site.

Change to:

a. These evaluation studies will utilize the fixed HD vapor durations that had been shown previously to produce a high incidence of microvesication. Ten animals per TX are evaluated for changes in erythema, edema, and histopathology at approximately 24 hr following HD exposure. TX and HD exposures are applied to the animals as indicated in the following schematic:



b. The dosing sites are randomized anteriorly to posteriorly for each animal to minimize experimental bias due to positional effects. Both the control and TX shams are always kept at the two anterior exposure sites or at the two posterior sites. All four TXs are evaluated concurrently, with animals from each group being exposed over a couple of days. Edema and histopathology measurements are made for each dose site.

Reason for Change: The initial study indicated that the treatment used produced a systemic as well as a local effect. The HD-control sites need to be performed using animals that are not treated with therapeutic.

Impact of Change: This change will result in additional animals being used for the repeat of this section of the study, but will increase the quality of data produced.

Change No. 3: Page 34, Section V.B.4.

Change from:

4. Total Number of Animals Required:

a. Mice - 386

(1) Dose Response Studies - 14 mice
(2) TX Evaluation Studies - 60 mice
(3) Endpoint Optimization -15 mice
(4) Module I Studies - 76 mice
(5) Module II Studies - 137 mice
(6) Module III Studies - 84 mice

b. Ninety additional mice are requested because of the uncertainty of the dose ranges for HD vapor cap exposures and the additional of evaluations at additional time points. Battelle's Institutional Animal Care and Use Committee (IACUC) procedures for requesting approval for additional animals will be followed

Change to:

4. Total Number of Animals Required:

a. Mice - 352

(1) Dose Response Studies - 14 mice
(2) TX Evaluation Studies - 110 mice
(3) Endpoint Optimization -15 mice
(4) Module I Studies - 76 mice
(5) Module II Studies - 137 mice

b. Ninety additional mice are requested because of the uncertainty of the dose ranges for HD vapor cap exposures and the additional of evaluations at additional time points. Battelle's Institutional Animal Care and Use Committee (IACUC) procedures for requesting approval for additional animals will be followed

Reason for Change: One of the treatment evaluation studies needs to be repeated as the systemic effect of the treatment affected the HD-control site. This results in 50 additional animals being used under this phase of the study. Animals will not be used under this protocol for Module III studies. Instead mouse ear specimens from animals exposed to liquid HD will be sent to the MREF from USAMRICD for biomarker analyses. The analyses with these specimens should be similar to that performed under Module II testing.

Impact on Study: These changes will decrease the number of animals used under this protocol and should also provide information regarding the correlation between the hairless mouse and haired mouse models.

Change No. 4: Page 35, Section V.C.1.

Change from:

C. Technical Methods:

1. Pain:

a. USDA (Form 18-3) Pain Category -

(1) No Pain - 90 mice

(2) Alleviated Pain - 386 mice

(3) Unalleviated Pain or Distress - 0 animals

Change to :

1. Pain:

a. USDA (Form 18-3) Pain Category -

(1) No Pain - 90 mice

(2) Alleviated Pain - 352 mice

(3) Unalleviated Pain or Distress - 0 animals

Reason for Change: A part of the treatment validation studies needs to be repeated and animals will no longer be required for Module III testing.

Impact on Study: The expected number of animals in category (2) is decreased by 32 animals.

Approvals:

James A. Blank, Ph.D., Study Director — Date 6/25/98

LTC Richard R. Stotts, D.V.M., Ph.D.
USAMRICD — Date 29 Jun 98

Reviewed and Registered by:

Elisha N. Morrison, M.S.
Quality Assurance Specialist — Date 7/2/98

Evaluation of Biomarkers for Sulfur Mustard Exposure in the Euthymic Hairless Mouse Model

MREF Protocol 118 (G155541A) Protocol Amendment No. 4

Change No. 1: The Study Director for this study is changed from Jim Blank, Ph.D. to Michele Danne, B.A.

Reason for Change: Jim Blank has left Battelle employment.

Impact on Study: The experimental phase of the study is 90 percent completed. There is no impact on the study.

Effective Date: 6/1/99

Approved by:

Michele Danne, B.A.
Study Director — Date: 7/1/99

James Estep, Ph.D., D.V.M.
Manager — Date: 7-1-99

LTC Richard R. Stotts, D.V.M., Ph.D.
USAMRICD COR — Date: 1 July 1999

Quality Assurance Review and Registration

Elisha N. Morrison, M.S.
Senior Quality Assurance Specialist — Date: 7/1/99

Evaluation of Biomarkers for Sulfur Mustard Exposure in the Euthymic Hairless Mouse Model

MREF Protocol 118 (G155541A) Protocol Amendment No. 5

Change No. 1: Page 9, Section V.A.5.d.

Delete:

d. No histopathology is performed in this module.

Reason for Change: Sentence was left in from original protocol and conflicted with amendment No. 2 which states that histopathology measurements are made for this module.

Impact on Study: There is no impact on the study.

Approved by:

Michele M. Danne, B.A. — Date: 4/27/00
Study Director

James E. Estep, D.V.M., Ph.D. — Date: 4-28-00
Manager

LTC Richard R. Stotts, D.V.M., Ph.D., D.A.B.T. — Date: 27 April 2000
USAMRICD COR

Quality Assurance Review and Registration

Elisha N. Morrison, M.S. — Date: 4/27/00
Senior Quality Assurance Specialist

APPENDIX B

MREF *In Vitro* Method No. 22

Method for Determining Protein Concentration Using the Pierce BCA Protein Assay

METHOD FOR DETERMINING PROTEIN CONCENTRATION USING THE PIERCE BCA PROTEIN ASSAY

A. Statement of Work: This method describes the measurement of protein concentration in a biological sample. The sample which contains the unknown amount of protein is incubated with the assay reagents, then the amount of color formed is measured spectrophotometrically using a microplate reader or other type of spectrophotometer. The amount of color formed is directly related to the amount of protein present in the sample. The protein concentration is determined from a series of standards containing known amounts of protein which are concurrently assayed with the sample.

B. Materials:

The BCA Protein Assay Reagent Kit is obtained from Pierce (Rockford, Il) and contains the following materials:

1. Albumin Standard (2.0 mg/mL; No. 23209)

2. Reagents A and B (No. 323225X)

C. Procedures:

1. Standard Preparation - The standards are prepared by diluting the stock standard provided in a sealed ampule with diluent (Millipore water). The stock standard is 2 mg/mL. Seven 15-mL tubes are labelled with the date of preparation, initial of individual making the dilution, task number, and the following information: 500 µg/mL albumin standard, 250 µg/mL albumin standard, 125 µg/mL albumin standard, 62.5 µg/mL albumin standard, 31.25 µg/mL albumin standard, 10 µg/mL albumin standard, and 0 µg/mL albumin standard. These standards may be used only once, but may be prepared in bulk quantity, aliquoted, and stored at -70 C for 12 months.

   a. A 500 µg/mL standard is prepared by adding 1000 µL of the 2 mg/mL stock standard to 3 mL of diluent. The tube is cappped and mixed by slowly inverting the tube back and forth 10 times.

   b. A 250 µg/mL standard is prepared by adding 3 mL of the 500 µg/mL standard to 3 mL of diluent. The tube is capped and mixed by slowing inverting the tube back and forth ten times.

   c. A 125 µg/mL standard is prepared by adding 3 mL of the 250 µg/mL standard to 3 mL of diluent. The tube is capped and mixed by slowing inverting the tube back and forth ten times.

   d. A 62.5 µg/mL standard is prepared by adding 3 mL of the 125 µg/mL standard to 3 mL of diluent. The tube is capped and mixed by slowing inverting the tube back and forth ten times.

e. A 31.25 µg/mL standard is prepared by adding 3 mL of the 62.5 µg/mL standard to 3 mL of diluent. The tube is capped and mixed by slowing inverting the tube back and forth ten times.

f. A 10 µg/mL standard is prepared by adding 1.6 mL of the 31.25 µg/mL standard to 3.4 mL of diluent. The tube is capped and mixed by slowly inverting the tube back and forth 10 times.

g. The last tube contains diluent only.

2. Assay Reagent Preparation - The assay reagent is prepared by mixing Reagent A and B together in a 49:1 ratio. Before removing reagents from any of the containers, first mix the individual reagent containers by a series of inversions. Once the reagents are combined, they must be used on the day of preparation. Any unused combined reagent is discarded. Assay Reagent may be prepared by adding the individual reagents as follows:

Total Volume (mL)	Reagent A (mL)	Reagent B (mL)
5	4.9	0.1
10	9.8	0.2
15	14.7	0.3
20	19.6	0.4

3. Protein Determination

a. The microplate diagram, as shown in Attachment A, identifies sample location on the microplate.

b. Twenty five microliters of each of the standards, in triplicate, are added to the appropriate wells of the microplates.

c. The protein sample is gently mixed and 25 µL of protein sample is added to the appropriate well of a 96-well microtiter plate. Duplicate analyses may be performed on each sample, and it may be necessary to dilute the sample so that the amount of protein in the diluted samples is between 10 and 250 µg/mL. The sample vehicle should be used to prepare all dilutions. The sample vehicle is also added to wells in duplicate to obtain a sample background value.

d. The Assay Reagent is mixed just prior to use. Two hundred microliters of Assay Reagent is added per well. The time of addition is noted on the form shown in Attachment A as well as on the cover of the microplate.

e. The microplate is mixed using a microplate shaker or by holding the plate on two sides with one hand and gently tapping a third

side with the other hand. Mixing should be performed for 30 seconds.

f. The microplate is transferred to a 37 C incubator for 30 min.

g. After the incubation, the plate is removed from the incubator and the lid carefully removed. Care is taken to prevent comtaminating wells with the condensate that will be on the inside of the lid. The absorbance of each loaded well at 540 nm or 570 nm (preferable 570 nm if the microplate reader is equipped with this filter, otherwise 540 nm will suffice) is obtained.

4. Calculations

a. The average zero (or background standard delta absorbance) value is subtracted from all other sample standard delta absorbance values. Linear regression is performed using protein concentration as the independent or X-variable and delta absorbance values minus background as the dependent or Y-variable.

b. The average background delta absorbance value is subtracted from sample delta absorbance values. Using an equation for a line, as shown below, with the variables (slope and Y-intercept) obtained from linear regression of the standards, the protein concentrations of the samples are determined.

$$X = \frac{(Y - b)}{m}$$

where,

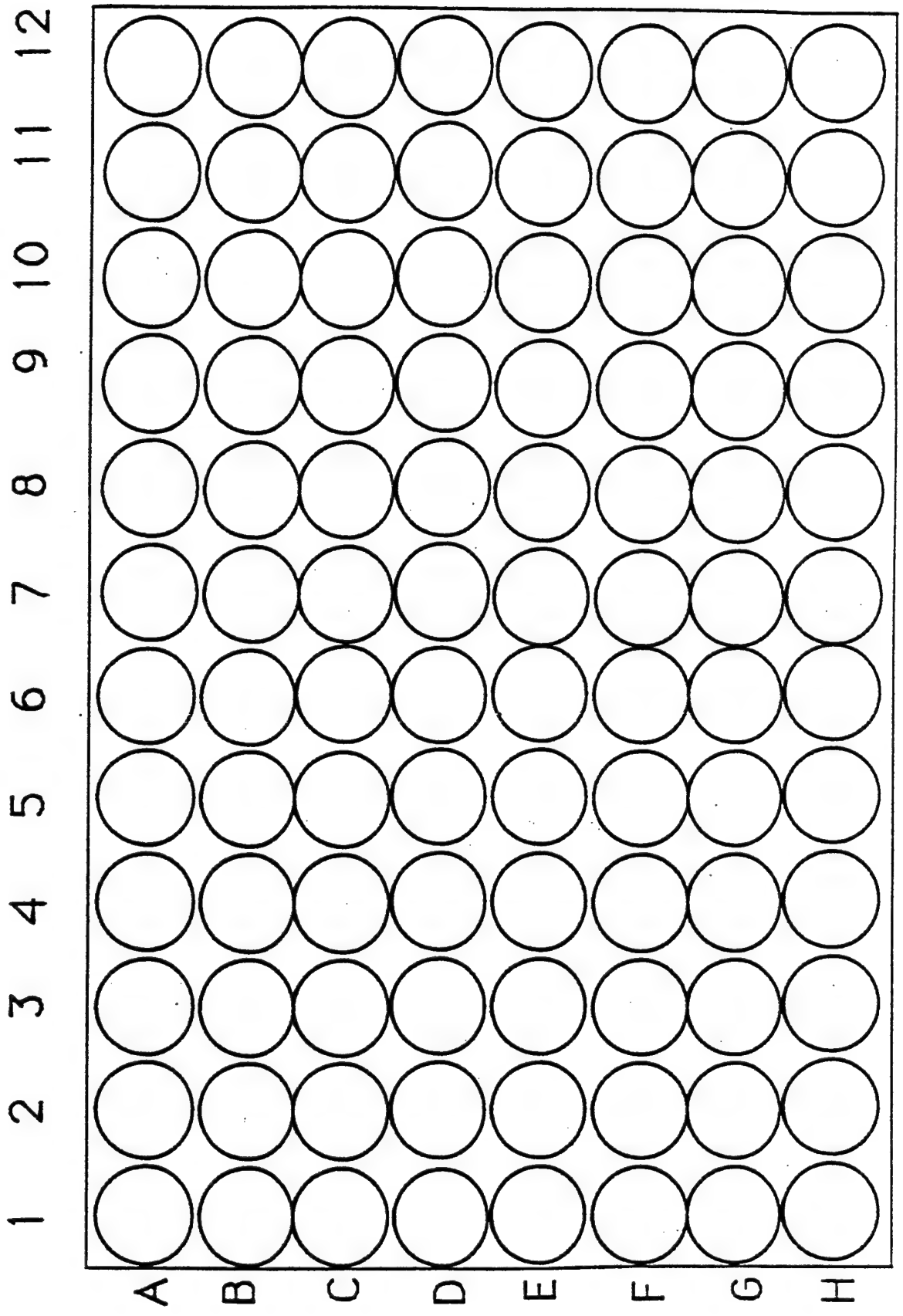
X = protein concentration (μg/mL)
Y = background subtracted delta absorbance value
m = slope
b = Y-intercept

c. If a diluted sample was analyzed, then the resulting protein concentration value obtained from Step b must be multiplied by the sample dilution factor.

Originated by: James R. L — Sept 24, 1993
James A. Blank, Ph.D. — Date
Principal Research Scientist

Reviewed by: Carl T. Olson — 9/24/93
Carl T. Olson, Ph.D. — Date
Senior Research Scientist

ATTACHMENT A



APPENDIX C

MREF *In Vitro* Method No. 49

Method for Extraction, Quantification and Electrophoresis of RNA from Mouse Skin Tissue

METHOD FOR EXTRACTION, QUANTIFICATION AND ELECTROPHORESIS OF RNA FROM MOUSE SKIN TISSUE

A. Statement of Work: This method is to be used for the extraction, quantification and electrophoresis of RNA from skin tissue biopsy samples obtained from mice. The method of extraction is based on the procedure of Chomczynski and Sacchi (1987) which relies on the formation of complexes of RNA with guanidinium and water molecules, and abolishes hydrophilic interactions of DNA and proteins thereby removing contaminating DNA and proteins.

B. Materials To Be Used:

1. Purchased from Tel-Test, Inc., Friendswood, TX:
   a. RNAzol B, Cat. No. CS-105
2. Purchased from FMC Corporation, Rockland, ME:
   a. RNase Decontamination Solution, Cat. No. 50576
3. Purchased from Sigma Chemical Co., St. Louis, MO:
   a. Isopropanol, Cat. No. I-9516
   b. Ethidium Bromide, Cat. No. E-1510
   c. Formaldehyde, Cat. No. F-8775
   d. Chloroform:Isoamyl Alcohol, Cat. No. C-0549
   e. 10X Tris-Borate-EDTA, Cat. No. T-4415
   f. Formamide, Cat. No. F-9037
   g. Diethyl Pyrocarbonate, Cat. No. D-5758
   h. 10X MOPS Buffer(3-[N-Morpholino]propanesulfonic acid) Cat. No. M-5755
4. Purchased from Life Technologies, Inc., Grand Island, NY:
   a. Agarose, Cat. No. 15510-019

b. 1mm, 20-tooth Delrin Comb, Cat. No. 41007-014

c. Gel Electrophoresis Apparatus, Cat. No. 21069-018

5. Deionized, distilled water (ddH_20) is prepared using a Milli-Q water purification system in conjunction with the Milli-R04 water purification system. Water of equilivent quality may be purchased and used.

C. Equipment: Freezer, refrigerator, labels, first-aid kit, weighing paper, wiping tissues, polypropylene snap-cap test tubes, Eppendorf microcentrifuge tubes, laboratory coat, safety shoes, safety glasses, disposable nitrile gloves, Eppendorf pipettors with pipette tips, vortex mixer, picofuge, Eppendorf refrigerated microcentrifuge, Mettler AE 100 balance, cryogenic vials, liquid nitrogen, Dewar for liquid nitrogen, cryo-safe gloves, Tekmar-Dorhman Tissuemizer with the 8 mm probe, ice buckets with ice, electrophoresis power supply unit, microcentrifuge tube racks, transilluminator, horizontal gel electrophoresis apparatus.

D. Procedures:

1. Work Area and Instrument Set-Up: The work area for sample-handling is a standard laboratory benchtop. All equipment must be treated using RNase Decontamination Solution. The Eppendorf model 5417R microcentrifuge is turned on and allowed to cool down to equilibrate to approximately 4C.

2. Reagent Preparation:

a. 1X MOPS Electrophoresis Buffer: Dilute 200 mL of 10X MOPS in 1800 mL of DEPC-treated H_20. The shelf life of this preparation is 6 months when stored at approximately 4C in the dark.

b. One percent Denaturing Agarose Gel: In a nuclease-free Erlenmeyer flask combine 30 mL 10X MOPS buffer, 3 g agarose, and 265 mL DEPC-treated water. Heat until dissolved. Add 5.3 mL formaldehyde and 12 μL of 10 μg/mL ethidium bromide solution. Allow this mixture to cool in a 65C water bath. Pour into the horizontal gel apparatus to solidify around a 1mm, 20-tooth Delrin comb.

c. Diethyl pyrocarbonate-treated water: Add 1 mL DEPC to 1 L of Milli-Q water. Allow solution to sit overnight at room temperature. Autoclave on liquid cycle for 20 minutes. The shelf life is 2 years when bottles remain unopened, and 6 months after opening.

3. Extraction and Precipitation of RNA: Previously harvested biopsy punch samples are stored in cryo-vials at below approximately –70C. When removed from the freezer for processing the tissue samples are maintained in liquid nitrogen.

   a. Pipet approximately 2.0 mL of RNAzol reagent into a polypropylene snap-cap tube for each tissue sample to be processed. One at a time, transfer the tissue samples to the tubes containing the RNAzol. Immediately begin homogenization using the Techmar-Dorhman Tissuemizer that has the 8 mm probe attached. Samples are homogenized for approximately 15 seconds.

   b. The homogenate is divided in half, each half being transferred to a nuclease-free Eppendorf tube that contains approximately 100 μL of chloroform:isoamyl alcohol (24:1). The samples are briefly vortexed, then incubated on ice for 10 minutes with intermittent vortexing. During this incubation period additional samples are homogenized.

   c. Homogenates are then centrifuged at approximately 17,900 x g (13,000 rpm), at approximately 4C for approximately 15 minutes in an Eppendorf Model 5417R microcentrifuge.

   d. The aqueous phase is transferred to an Eppendorf tube that contains approximately 500 μL chloroform:isoamyl alcohol (24:1). Tubes are vortexed briefly and then centrifuged at approximately 17,900 x g (13,000 rpm), at 4C for approximately 5 minutes.

   e. Again the aqueous phase is transferred to an Eppendorf tube that contains approximately 550 μL of isopropanol.

   f. Samples are allowed to precipitate overnight at below approximately –20C.

4. Washing and Resuspension:

   a. The precipitated samples are centrifuged at approximately 17,900 x g (13,000 rpm), 4C for approximately 20 minutes in an Eppendorf Model 5417R microcentrifuge.

   b. The supernatant is removed and the RNA pellet washed using approximately 200 μL of cold 70% ethanol followed by approximately 200 μL of cold 100% ethanol.

   c. The RNA samples are air-dried to allow residual ethanol to evaporate.

d. The RNA pellets are resuspended in approximately 100 μL of DEPC-treated water and stored until a spectrophotometric reading is desired, and/or an agarose gel is to be run to check the integrity of the ribosomal bands.

5. Spectrophotometry:

An aliquot from each RNA sample is diluted 1/10 in DEPC-treated water to a final volume of 1000 μL. The OD 260/280 is read using a spectrophotometer. Data is recorded on Form No. MREF *InVitro*-097.

6. Electrophoresis:

a. A 1% agarose gel is prepared as indicated above.

b. Electrophoresis buffer consisting of 1X MOPS is prepared in DEPC-treated water as indicated above.

c. Gel loading buffer is prepared as follows:
20 μL of 10X MOPS
55 μL of 37% formaldehyde
90 μL of formamide
15 μL of bromophenol blue dye mixture

d. Approximately 5 μL of RNA is diluted with approximately 5 μL of DEPC-treated water in nuclease-free Eppendorf tubes. Approximately 13 μL of gel loading buffer is added to each sample. Samples are denatured in a water bath set at approximately 80 C for approximately 2 minutes. Samples are cooled on ice prior to being loaded on a gel for electrophoresis.

e. Electrophorese at 110V for approximately 2.5 hours, monitoring the dye front as it progresses through the gel.

f. After the samples have run long enough for sufficient separation of the banding pattern, shut off the power unit and transfer the gel over to the transilluminator. Wearing UV safe glasses, view the gel and note the appearance of the banding pattern. Record observations on Form No. MREF *InVitro*-097.

g. A polaroid photo and/or a digital image can be taken for documentation purposes.

E. References:

1. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156-159: Single-Step Method of RNA Isolation by Acid Guanidinium Thiocyanate-Phenol-Chloroform Extraction.
2. Sambrook, J., E. F. Fritsch, and T. Maniatus. 1989. Molecular Cloning: A Laboratory Manual, Second Edition (N. Ford, C. Nolon, M. Ferguson, eds), Cold Spring Harbor Laboratory Press, New York.

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APPENDIX D

MREF *In Vitro* Method No. 54

Method for the Ribonuclease Protection Assay

Method For The Ribonuclease Protection Assay

A. Statement of Work: This method is to be used for the detection and quantitation of messenger ribonucleic acid (mRNA) species. The procedure is based on the enzymatic ability to generate high-specific-activity ^{32}P-labeled antisense ribonucleic acid (RNA) probes via T7 polymerase-directed *in vitro* transcription of deoxyribonucleic (DNA) template fragments. The templates represent a portion of the sequence from the mRNA species that is being investigated. By assembling several different templates into biologically relevant sets, multiple mRNA species can be simultaneously quantified from an RNA sample allowing for comparative analysis. The radiolabeled probe set is hybridized in excess to the sample RNA, after which unhybridized probe and other single-stranded RNA are enzymatically removed. The remaining "RNase-protected" duplexes are purified, resolved on denaturing polyacrylamide gels, and quantified by autoradiography.

B. Materials To Be Used:

1. Purchased from NEN Life Science Products, Boston, MA:
   a. [Alpha-^{32}P]UTP, Cat. No. BLU-007H
2. Purchased from Pharmingen, San Diego, CA:
   a. *In vitro* Transcription Kit, Cat. No. 45004K
   b. Multi-Probe Template Set, Cat. No. (varies)
   c. RPA Kit, Cat. No. 45014K
3. Purchased from Sigma Chemical Co., St. Louis, MO:
   a. Exposure Cassette, Cat. No. E-9510
   b. Sigmacote, Cat. No. SL-2
   c. Chloroform:IAA, Cat. No. C-0549
   d. 10X Tris-Borate-EDTA, Cat. No. T-4415
   e. Phenol, Cat. No. P-4557
   f. Phenol Buffer, Cat. No. B-5658

g. Mineral Oil, Cat. No. M-5904

h. Intensifying Screen, Cat. No. Z35,700-6

i. X-Ray Film, Cat. No. Z35, 858-4

4. Purchased from Eastman Kodak Co., New Haven, CT:

a. Ethanol, Molecular Grade, Cat. No. IB15720

5. Purchased from National Diagnostics, Atlanta, GA:

a. Ammonium Persulfate, Cat. No. EC-504

b. Sequagel Buffer, Cat. No. EC-835

c. Sequagel Concentrate, Cat. No. EC-830

d. Sequagel Diluent, Cat. No. EC-840

6. Purchased from Bio-Rad, Hercules, CA:

a. TEMED, Cat. No. 161-0800

b. Sequencing Filter Paper, Cat. No. 165-0959

c. Model 583 Gel Dryer, Cat. No. 165-1745

d. Power Pack Model 3000, Cat. No. 165-5056

e. Sequencing Apparatus, Cat. No. 165-3861

f. 20-Tooth Comb, Cat. No. 165-3686

g. Glass Plates, Cat. No. 165-3646

7. Purchased from USA Scientific, Ocala, FL:

a. Tiplet Rad-Tip Box, Cat. No. 3056-6000

b. Beta Rack, Cat. No. 3040-0000

c. Angled Shield, Cat. No. 3051-0000

d. Microcentrifuge Tubes, Cat. No. 1415-2600

8. Purchased from Rainin, Woburn, MA:

a. FinePoint Aerosal Resistant Tips, Cat. No. RT-10GF, HR-200F

b. Sterile Pipet Tips, Cat. No. RT-10GS, HR-2505S, HR-1000S

9. Purchased from Fisher Scientific, Pittsurgh, PA

a. Polypropylene Lab Wrap, Cat. No. 15-610

C. Equipment: Freezer, refrigerator, label tape, first-aid kit, weighing paper, wiping tissues, laboratory coat, Ludlum Geiger Counter, Beckman LS 3801 Liquid Scintillation Counter, safety shoes, safety glasses, disposable nitrile gloves, Eppendorf pipettors, vortex mixer, picofuge, Mettler AE 100 balance with calibration standards, ice buckets with ice, metal spatulas, plastic weigh boats, polystyrene pipets with pipettor, microcentrifuge tube racks, safelight, X-ray film developer, Model 5417 Eppendorf refrigerated microcentrifuge and rotor, dry heat block, scintillation vials.

D. Procedures:

1. Work Area and Instrument Set-Up: The work area for sample handling is a clean laboratory benchtop that is suitable for working with nucleic acids. The radioisotope is handled in a Class II ventilated hood and is monitored with a hand-held Geiger Counter.

2. Reagent Preparation: 0.25X Tris-Borate-EDTA: Dilute approximately 25 mL of 10X TBE with approximately 475 mL of sterile, distilled water. The shelf life of this preparation is approximately 6 months when stored at approximately 4C.

3. Preparation of the Labeled Anti-Sense Riboprobe:

   a. Bring the [alpha-^{32}P]UTP, GACU nucleotide pool, DTT, 5X transcription buffer, and RiboQuant™ template set to room temperature. For each probe synthesis, add approximate amounts of the following, in order, to a microcentrifuge tube:

      1 µL RNasin = 40 U
      1 µL GACU pool = 2.75 mM GAC, 61 mM U
      2 µL DTT = 100 mM
      4 µL 5X Transcription Buffer
      1 µL RPA Template Set = 50 ng
      10 µL [alpha-^{32}P]UTP = 100 µCi
      1 µL T7 polymerase = 20 U

      Pipette to mix, then picofuge to collect the contents.
      Incubate at approximately 37C for approximately 1 hr.

   b. Stop the reaction by adding approximately 2 µL of DNase (2 U). Flick the tube to mix, then picofuge. Incubate at approximately 37C for approximately 30 min.

   c. Add the approximate amounts of the following reagents, in order, to the reaction mix:

      26 µL 20 mM EDTA
      25 µL Tris-sat phenol
      25 µL chloroform:IAA
      2 µL yeast tRNA = 4 µg

   d. Vortex into an emulsion and centrifuge for approximately 5 min at approximately 15000 x g, at room temperature.

   e. Transfer the aqueous phase to a new microcentrifuge tube that contains approximately 50 µL chloroform:IAA. Vortex, then centrifuge for approximately 2 min at approximately 15000 x g, at room temp.

   f. Transfer the aqueous phase to a new microcentrifuge that contains approximately 50 µL 4M ammonium acetate and 250 µL cold absolute ethanol. Mix and incubate for approximately 1 hr at approximately -20C.

   g. Centrifuge for approximately 20 min at 15000 x g at approximately 4C.

h. Remove the supernatant to waste and air dry the pellet for approximately 5 min. Add approximately 50 µL of Hybridization Buffer and solubilize the pellet by gently vortexing for approximately 20 sec. Picofuge to collect contents.

i. Quantitate approximately 1 µL of the solubilized riboprobe with the Beckman 3801 Liquid Scintillation counter. Typical yields range from 30,000 to 3 million cpm/µL using Cherenkov counting. Store the probe at approximately -20C for use in the overnight hybridizations.

4. Hybridization Protocol: This protocol outlines the steps required to hybridize the previously labeled riboprobe in excess to the RNA samples of interest. This requires that the organically extracted nucleic acid samples be precipitated with ethanol and air-dried.

a. Add approximately 8 µL of Hybridization Buffer to each RNA sample. Solubilize the nucleic acid pellets by gently vortexing for approximately 2 min, then picofuge to collect the contents.

b. Dilute the riboprobe to the appropriate concentration (mCK-2 = 30,000 cpm/µL) using Hybridization Buffer.

c. Add approximately 2 µL of diluted probe to each RNA sample and mix by pipetting.

d. Add a drop of mineral oil to each tube and picofuge.

e. Place the samples in a heat block pre-warmed to approximately 90C. Immediately turn the temperature to approximately 56C.

f. Incubate the samples for approximately 12-16 hours.

5. RNase Treatments

a. Prepare the RNase cocktail (approximate quantities per 24 samples):

2.5 mL RNase Buffer
6 µL RNase A + T1 mix

Remove the RNA samples from the heat block and pipet approximately 100 µL of the RNase cocktail underneath the oil. Picofuge briefly to separate the phases. Incubate for approximately 45 min at approximately 30C.

b. Before the RNase digestion is completed, prepare the Proteinase K cocktail (approximate quantities per 20 samples):

390 µL Proteinase K Buffer
30 µL Proteinase K = 300 µg
30 µL yeast tRNA = 60 µg

Mix and add approximately 18 µL of the cocktail to new microcentrifuge tubes.

c. After approximately 45 min, extract the digests from underneath the oil and transfer to the tubes containing the Proteinase K solution. Vortex briefly and picofuge. Incubate for approximately 15 min at approximately 37C.

d. Add approximately 65 µL Tris-sat phenol and 65 µL chloroform:IAA to each sample. Vortex into an emulsion and spin for approximately 5 min at approximately 15000 x g at room temp.

e. Carefully transfer the aqueous phase (set pipettor to approximately 120 µL) to a new microcentrifuge that contains approximately 120 µL 4 M ammonium acetate and 650 µL cold ethanol. Mix gently and picofuge. Incubate samples for approximately 1 hr at approximately -20C.

f. Spin for approximately 15 min at approximately 15000 x g at approximately 4C. Carefully remove the supernatant and air-dry the pellets completely.

g. Add approximately 5 µL of 1X Loading Buffer. Vortex for approximately 2 min and picofuge to collect contents.

h. Prior to loading the samples on the gel, heat the samples for approximately 3 min at approximately 90C then place on ice.

6. Gel Resolution of Protected Probes

a. Prepare the sequencing gel plates by cleaning with water followed by ethanol. Siliconize the short plate. Assemble the apparatus using 0.4 mm spacers.

b. Combine the approximate amounts of the following to prepare a gel with a final concentration of approximately 5% acrylamide:

35 mL Diluent (urea)
10 mL Concentrate (acrylamide)
5 mL 10X Tris-Borate-EDTA
400 µL 10% Ammonium Persulfate
20 µL TEMED

c. Immediately inject into the gel mold and add the appropriate comb. Allow to polymerize for approximately 1 hour.

d. Remove the comb and flush the wells thoroughly with 0.5X TBE.

e. Prerun the gel at approximately 50 watts constant power for approximately 45 min in 0.5X TBE. Gel temp should be approximately 50C.

f. Denature the samples for approximately 3 min at approximately 90C then immediately place on ice.

g. Flush the wells again with 0.5X TBE.

h. Load the samples. In the first lane load a dilution of the probe set in loading buffer (approximately 3000 cpm/lane in 5 µL total volume) to serve as size markers.

i. Run the gel at approximately 50 watts constant power until the leading edge of the dye front reaches 30 cm.

j. Disassemble the gel apparatus, removing the short plate. Adsorb the gel to filter paper. Cover the gel with saran wrap. Place in the gel dryer under vacuum for approximately 1 hour at approximately 80C.

k. Place the dried gel on film in a cassette equipped with an intensifying screen to expose at -70C.

E. References:

1. Gilman, M. 1993. Ribonuclease protection assay. In *Current Protocols in Molecular Biology*, Vol. 1 (Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J. G. Seidmen, J.A. Smith and K. Stuhl, eds.), pp. 4.7.1-4.7.8. John Wiley and Sons, Inc., New York.

2. Sambrook, J., E.F. Fritsch and T. Maniatis. 1989. Synthesis of RNA probes by *in vitro* transcription of double-stranded DNA templates by bacteriophage DNA-dependent RNA polymerases. In *Molecular Cloning: A Laboratory Manual*, second edition (N. Ford, C. Nolan and M. Ferguson, eds.). Cold Spring Harbor Laboratory Press, New York, pp. 10.27-10.37.

3. Pharmingen. 1998. RiboQuant Multi-Probe RNase Protection Assay System Instruction Manual, fifth edition.

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APPENDIX E

MREF *In Vitro* Method No. 55

Method for Preparation and Determination of Myeloperoxidase in Hairless Mouse Skin Homogenate

METHOD FOR PREPARATION AND DETERMINAITON OF MYELOPEROXIDASE IN HAIRLESS MOUSE SKIN HOMOGENATE

A. Purpose: Myeloperoxidase (MPX) is an enzyme that is found in high concentrations in neutrophils. During an inflammatory process, neutrophils may be a very early response (~ couple hrs) element to the site of damage and may last for ~24 hrs. Measurement of MPX activity in homogenates prepared from skin biopsies is thought to be a good indicator of neutrophilic influx. MPX resides in the granules of neutrophils and has been reported somewhat difficult to solubilize. It has been reportec that homogenization of cells in the presence of hexadecyltrimethyl ammonium bromide (HTAB) followed by consecutive freeze/thaw cycles and sonication is effective for solubilizing MPX activity from the homogenate pellet fraction to the supernatant fraction (Bradley et al., 1982. J Invest Dermatol 78:206-209).

B. Reagents:

1. Potassium Phosphate (50 mM) - Prepared by adding 6.81 gm of potassium phosphate, monobasic to ~800 ml of Millipore water. The pH is adjusted to 6.0 and the solution is brought to volume (1000 ml).

2. HTAB Solution: 0.5% Solution - Add 0.05 gm of hexadecyltrimethyl ammonium bromide (HTAB) to 10 ml of the 50 mM potassium phosphate buffer (pH 6.0).

3. O-Dianisidine (0.19 mg/ml) - Prepared by adding 0.19 mg for every ml of 50 mM potassium phosphate buffer (pH 6.0). The material is weighed in a ventilated balance enclosure and the solution is stored protected from light (e.g., amber vial or container wrapped with aluminum foil.

4. Hydrogen Peroxide solutions: These are prepared under reduced lighting just prior to use, and should be discarded and not used after 2 hr.

   a. Working Stock Hydrogen Peroxide solution (0.3%): Prepared from a 3% hydrogen peroxide solution (e. g. 0.2 ml into 1.8 ml 50 mM potassium phosphate buffer, pH 6.0).

   b. 0.02% Hydrogen Peroxide Reagent Mix is prepared by adding 0.4 ml of Working stock hydrogen peroxide to 5.6 ml of the 0.19 mg/ml o-dianisidine solution.

   c. 0.00025% Hydrogen Peroxide Reagent Mix is prepared by adding 0.5 ml of 0.02% Hydrogen Peroxide Reagent Mix to 40 ml of the 0.19 mg/ml o-dianisidine solution.

5. Standard Preparation (Stock is 20 U/ml): Calbiochem cat #475911

1. 1 U/ml - Add 50 ul of the 20 U/ml stock to 950 ul of diluent.
2. 0.5 U/ml - Add 0.750 ml of the 1 U/ML Standard (# 1) to 0.750 ml of diluent.
3. 0.25 U/ml - Add 0.750 ml of the 0.5 U/ml Standard (#2) to 0.750 ml of diluent.
4. 0.125 U/ml - Add 0.750 ml of the 0.25 U/ml Standard (#3) to 0.750 ml of diluent
5. 0.0833 U/mL-Add 0.250 mL of the 0.125 U/mL Standard (#4) to 0.375 mL of diluent
6. 0.0625 U/ml - Add 0.5 ml of the .125 U/ml Standard (#4) to 0.5 ml of diluent.
7. 0.0313 U/ml - Add 0.5 ml of the 0.0625 U/ml Standard (#6) to 0.5 ml of diluent.
8. 0.0156 U/ml - Add 0.5 ml of the 0.0313 U/ml Standard (#7) to 0.5 ml of diluent.
9. diluent is used as the background

diluent = 50 mM potassium phosphate buffer, pH 6.0.

C. Tissue Treatments: The starting sample is a pellet from an approximately 12 mm biopsy punch of euthymic hairless mouse skin that has been snap-frozen in liquid nitrogen, pulverized using a Biopulverizer (Daigger), then solubilized in approximately 2.0 ml of phosphate buffered saline (PBS) and centrifuged for 30 min (50,000xg). The supernate is poured off and the test-tube containing the pellet is covered with parafilm and placed in -70C freezer until sample preparation.

D. MPX Sample Preparation:

1. Pellets are removed from the freezer and placed on ice. 1.5 ml of 0.5 % HTAB is added, and each sample is given a quick vortex.

2. Samples are then sonicated for 15 seconds using a probe sonicator. Caution is required to not overheat the tissues samples.

3. Each pellet is then subjected to 3 freeze/thaw cycles using liquid nitrogen and cool water baths. The samples are then resonicated for 15 sec.

4. Each sample is centrifuged at 50,000 x g for 30 minutes. The supernate is removed and placed in -70C freezer for later activity determinations.

E. MPX Activity Determination:

1. 20 ul of sample is added to 180 ul of the 0.00025% Hydrogen Peroxide Reagent Mix under reduced lighting.

2. After adding of the reagent mix the microplate is read at OD450 at 15 second intervals for a total of 3.0 minutes (13 readings total).

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(1) Note: Correction date should have been 5/19/99. EMM 5/24/99

APPENDIX F

MREF *In Vitro* Method No. 56

Method for Determining Serum Amyloid P (SAP) Levels in Hairless Mouse Skin Homogenate

METHOD FOR DETERIMING SERUM AMYLOID P (SAP) LEVELS IN HAIRLESS MOUSE SKIN HOMOGENATE

A. Assay Principle:

Acute phase reactive protein are proteins that are primarily synthesized and released by hepatic tissue in response to an inflammatory condition. SAP is a major acute phase reactive protein in mice, that can be measured using an enzyme immunoassay (EIA) technique.

B. Solution Preparation:

1. COATING BUFFER (CB) FOR ELISA PLATES: 10mM Tris (1.21 gm per liter), 140 mM NaCl (8.18 gm per liter), adjust pH to approximately 7.4 at room temperature. This solution can be stored at room temperature for 3 months from the date of preparation.

2. BLOCKING BUFFER: CB with 0.1% Gelatin (0.1 gm per every 100 mL). This solution can be used for 3 months from the date of preparation.

3. 5X WASH BUFFER: 50mM Tris (6.06 gm per liter), 0.7 M NaCl (40.91 gm per liter), 0.01% Sodium Azide (0.1 gm per liter), 0.25% Tween-20(2.5 mL per liter), adjust pH to 7.4. One volume of 5X wash buffer is diluted with 4 volumes of water prior to use. These buffers, 5X or 1X, can be stored at room temperature for 3 months from the date of preparation of the 5X buffer. ***The 5X buffer should never be used for washing plates.***

4. DILUTION BUFFER: CB with 1% bovine serum albumin (1 gm per every 100 mL) suitable for EIA procedures. The buffer should be kept, refrigerated, and may be stored for 3 months from the date of preparation.

C. Preparation Of Standards:

1. SAP Standards: series of dilutions of the SAP standard stock (100 µg/mL; Calbiochem Cat. #565193) are prepared for analysis.

2. Prepare a working stock by adding 10 ul of the standard stock to 2 mL of Dilution Buffer (500 ng/mL).

   a. 40 ng/mL - Add 480 ul of working stock to 5.5 mL of Dilution Buffer
   b. 20 ng/mL - Add 3 mL of Standard 1 (40 mg/mL) to 3 mL of Dilution Buffer
   c. 10 ng/mL - Add 3 mL of Standard 2 (20 mg/mL) to 3 mL of Dilution Buffer
   d. 5 ng/mL - Add 3 mL of Standard 3 (10 mg/mL) to 3 mL of Dilution Buffer

e. 2.5ng/mL - Add 3 mL of Standard 4 (5 mg/mL) to 3 mL of Dilution Buffer
f. 1.25 ng/mL - Add 3 mL of Standard 5 (2.5 mg/mL) to 3 mL of Dilution Buffer
g. 0.625 ng/mL - Add 3 mL of Standard 6 (1.25 mg/mL) to 3 mL of Dilution Buffer
h. 0.0 - 4 mL of Dilution Buffer

D. Dilution Of Antibody Reagents:

1. Primary Antibody (Affinity-Purified Sheep Anti-Mouse SAP Antibody): The volume of solution needed to coat the EIA plates (100 µl/well) is calculated. The antibody is diluted in COATING BUFFER to a concentration of 4 µg/mL to make an approximate 5 percent excess of what is needed. This is equivalent to 230 µl of stock antibody solution for every 25 mL of COATING BUFFER. The antibody concentration used may vary depending upon lot of antiserum from which the Ig are isolated.

2. Secondary Antibody (Rabbit Anti-Mouse SAP) (Calbiochem Cat# 565192): The volume of solution needed for the EIA plates (100 µl/well) used on that day is calculated. The antibody is diluted at 1:4000 in DILUTION BUFFER to make an approximate 5 percent excess of that needed. This is equivalent to 6.3 µl of stock antibody solution for every 25 mL of DILUTION BUFFER.

3. Tertiary or detection Antibody (Horseradish Peroxidase-Goat Anti-Rabbit IgG; Sigma Cat# A6667): The volume of solution needed for the ELISA plates (100 µl/well) used on that day is calculated. The antibody is diluted at 1:2000 in DILUTION BUFFER to make an approximate 5 percent excess of that needed. This is equivalent to 12.5 µl of stock antibody solution for every 25 mL of DILUTION BUFFER

E. EIA Procedure:

1. Affinity-purified sheep anti-mouse SAP antibody is diluted in COATING BUFFER to a final protein concentration of approximately 4 µg/mL.

2. 100 µl of the diluted SAP antibody prepared in step 1 is added per well of the Immulon II ELISA plate (Corning). The plates are covered with parafilm, refrigerated, and incubated overnight.

3. On the day of use, 200 µl/well of BLOCKING BUFFER is added, the plate covered with parafilm and incubated at room temperature on a shaker for approximately 60 min.

4. The wells are washed with 1X WASH BUFFER (Aspirate and fill wells completely four times).

5. Add 100 µl of dilution buffer, SAP standards or hairless mouse skin homogenate. The hairless skin homogenate is prepared in dilution buffer after being thawed at room temperature. Then the samples are added to the plates (e. g. in triplicate). The plates are covered with parafilm and incubated at room temperature on a shaker for 3 hrs. The plates are washed as in Step 4.

6. 100 µl/well of rabbit anti-mouse SAP is added to each well, the plates are covered and incubated on a shaker at room temperature for 1 hr. The plates are washed again per Step 4.

7. 100 µl/well of a horse-radish peroxidase conjugated goat anti-rabbit IgG is added, the plates are covered and incubated for 1 hr. at room temperature. The plates are washed again per Step 4.

8. 100 µl of 1-Step-Turbo TMB EIA reagent (3, 3', 5, 5', Tetramethyl Benzidine; Pierce Cat # 34022) is added under reduced light and the plates are incubated in the dark for 20 min. Do not cover the plates. 100 µl of dilute (2 M) sulfuric acid is added to stop the reaction. The plates are read at 450 nm.

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APPENDIX G

Module I Statistical Analyses

Battelle
. . . Putting Technology To Work

Project Number G1555-41ASTAT

Internal Distribution

Project Files
B. Pierce
B. Wood
J. Holdcraft
N. Niemuth
RMO

s:\niem\mref\Task 41\Module I cover memo + report doc

Date August 1, 2000

To Carol Sabourin

From Nancy Niemuth

Subject **Statistical Analysis of Study G1555-41A**
MREF Task 95-41 -- Module I

The attached report describes the statistical analysis of tissue weight, SAP, IL-6, MPX, and IL-1α data collected under Module I of MREF Task 95-41. Since no drug treatments were tested, the two-stage modeling approach used in Modules II and III was not applied in this module. The model fitted to the Module I data corresponds to 'Model 3' in the other reports. An electronic copy of the statistical report will be provided for use in preparing the final report on this study.

Please call me at (614)424-3231 if you have any questions.

NAN:lnl

For Review and Approval

	Name	Initials	Date
Originator	Nancy Niemuth	N	8/3/00
Concurrence	Jennifer Holdcraft	JH	
	Brandon Wood	BW	8/1/00
Approved	Bill Rosebrough	WRR	8/3/00

Sent Via:

Statistical Report for MREF Task 95-41 Module I Data

July 31, 2000

Introduction

Experiments were conducted under MREF Task 95-41 Module I to determine the effects of HD exposure at 2, 6, and 24 hours post-exposure in the euthymic hairless mouse model. Four exposure sites were tested on the back of each animal. Two sites, either A and D or B and C, were exposed to HD vapor for 6 minutes; The remaining sites on each animal were sham sites. Eight animals were tested at each post-exposure time. The average response of the two HD sites or two sham sites within each animal was used as the endpoint for statistical analysis. The endpoints evaluated were tissue weight, SAP, IL-6, MPX, and IL -1α.

Statistical Methods

The IL-6 data in Module I included several negative responses ranging from –0.0009 to –0.064. To avoid introducing bias to the statistical analysis, these values were not changed, although it is recognized that IL-6 levels cannot fall below zero.

An analysis of variance (ANOVA) model was used to analyze each endpoint. For each animal, the response variable was calculated as the difference between the HD-exposed site mean and sham site mean. The ANOVA models took the following form:

$$Y_{ij} = \mu + \beta_i + \varepsilon_{ij},$$

where Y_{ij} = tissue weight, SAP, IL-6, MPX, or IL -1α response for j^{th} animal at the i^{th} post-exposure time
μ = overall average value of the response
β_i = effect of i^{th} post-exposure time
ε = uncontrolled variation.

The ANOVA models were fitted using the SAS (V8) MIXED procedure. Model parameters were used to estimate the difference between HD-exposed and sham site means. In addition, Tukey's multiple comparison procedure was used to compare the effects of HD at the three post-exposure times.

In addition, the relative response at each post-exposure time was calculated as

Relative Response = 100 * (HD – SHAM)/ SHAM

where SHAM and HD are the means for sham sites and HD-exposed sites, respectively.

Results

Descriptive statistics and relative response compared to the sham sites for tissue weight, SAP, IL-6, MPX, and IL -1α are displayed in Tables 1-5, respectively. Model estimated differences between HD-exposed sites and sham sites at each post-exposure time for all endpoints are presented in Table 6. Full data listings for Module I analyses are provided in Appendix A.

A statistically significant increase in tissue weights was associated with HD exposure at both 6 and 24 hours post-exposure (Tables 1 and 6), while tissue weights did not change on average at 2 hours post-exposure. The increase in tissue weights between HD-exposed sites and sham sites at 24 hours post-exposure was significantly greater than that at 2 hours and 6 hours post-exposure (Table 6).

SAP levels at 24 hours post-exposure were significantly increased as a result of HD-exposure, but were not significantly changed at 2 or 6 hours post-exposure (Tables 2 and 6). The difference between HD-exposed sites and sham sites was significantly greater at 24 hours post-exposure than at 2 or 6 hours post-exposure (Table 6). Note that SAP levels in sham sites at 24 hours were approximately double those at 2 and 6 hours.

IL-6 values at HD exposed sites were significantly increased at 6 hours post-exposure, but there was no significant difference between HD-exposed sites and sham sites at 2 or 24 hours post-exposure (Tables 3 and 6). The difference between HD-exposed and sham sites at 6 hours post-exposure was significantly greater than the differences at 2 and 24 hours post-exposure (Table 6).

A statistically significant increase in MPX levels was associated with HD-exposure at 24 hours post-exposure, while the differences between HD-exposed and sham sites at 2 and 6 hours post-exposure were not statistically significant (Tables 4 and 6). The difference between HD-exposed sites and sham sites was significantly greater at 24 hours post-exposure than at 2 or 6 hours post-exposure (Table 6).

A statistically significant decrease in IL-1α levels was found at HD-exposed sites at 24 hours post-exposure, while the differences between HD-exposed and sham sites at 2 and 6 hours post-exposure were not statistically significant (Tables 5 and 6). The decrease between HD-exposed and sham sites at 24 hours post-exposure was significantly different than the estimated differences at 2 and 6 hours post-exposure (Table 6).

Conclusions

Exposure to HD was associated with significant increases in tissue weight at 6 and 24 hours post exposure, SAP at 24 hours post-exposure, IL-6 at 6 hours post-exposure, and MPX at 24 hours post-exposure, and a significant decrease in IL-1α at 24 hours post-exposure. For tissue weight, SAP, MPX and IL-1α the difference between HD-exposed sites and sham sites at 24 hours post-exposure was significantly different from the respective differences at 2 and 6 hours post-exposure, while for IL-6 the difference at 6 hours post-exposure was significantly different from those at 2 and 24 hours post-exposure.

Table 1. Descriptive Statistics for Tissue Weight by Time Post-Exposure and Exposure.

Time Post-Exposure	Exposure	Tissue Weight (g)			
		Number of Animals	Mean	SD	Relative Response[1]
2 hr	Sham	8	0.097	0.008	0
	HD	8	0.097	0.008	
6 hr	Sham	8	0.089	0.018	8
	HD	8	0.096	0.012	
24 hr	Sham	8	0.092	0.008	33
	HD	8	0.122	0.006	

[1]Relative Response = 100*(HD-SHAM)/SHAM.

Table 2. Descriptive Statistics for SAP by Time Post-Exposure and Exposure.

Time Post-Exposure	Exposure	SAP (ng/mg/mg)			
		Number of Animals	Mean	SD	Relative Response[1]
2 hr	Sham	8	0.066	0.067	26
	HD	8	0.083	0.088	
6 hr	Sham	8	0.086	0.040	6
	HD	8	0.091	0.042	
24 hr	Sham	8	0.169	0.173	84
	HD	8	0.312	0.318	

[1]Relative Response = 100*(HD-SHAM)/SHAM.

Table 3. Descriptive Statistics for IL-6 by Time Post-Exposure and Exposure.

Time Post-Exposure	Exposure	IL-6 (pg/mg/mg)			
		Number of Animals	Mean	SD	Relative Response[1]
2 hr	Sham	8	0.030	0.032	66
	HD	8	0.049	0.052	
6 hr	Sham	8	0.133	0.248	730
	HD	8	1.100	0.751	
24 hr	Sham	8	0.048	0.044	304
	HD	8	0.194	0.089	

[1]Relative Response = 100*(HD-SHAM)/SHAM.

Table 4. Descriptive Statistics for MPX by Time Post-Exposure and Exposure.

Time Post-Exposure	Exposure	MPX (U/mg)			
		Number of Animals	Mean	SD	Relative Response[1]
2 hr	Sham	8	0.138	0.063	-14
	HD	8	0.119	0.055	
6 hr	Sham	8	0.159	0.097	21
	HD	8	0.194	0.055	
24 hr	Sham	8	0.155	0.058	119
	HD	8	0.339	0.138	

[1]Relative Response = 100*(HD-SHAM)/SHAM.

Table 5. Descriptive Statistics for IL-1α by Time Post-Exposure and Exposure.

Time Post-Exposure	Exposure	IL-1α (pg/mg/mg)			
		Number of Animals	Mean	SD	Relative Response[1]
2 hr	Sham	8	6.959	1.084	7
	HD	8	7.480	2.172	
6 hr	Sham	8	8.729	2.756	-10
	HD	8	7.845	2.622	
24 hr	Sham	8	8.520	3.722	-52
	HD	8	4.069	1.241	

[1]Relative Response = 100*(HD-SHAM)/SHAM.

Table 6. Model Estimated Difference betweeen HD-Exposed Sites and Sham Sites at each Post-Exposure Time for Module I Endpoints.

Endpoint	Time Post-Exposure	Estimated Difference (HD-Sham)	SE	p-Value (T-Test)
Tissue Weight	2 hr [24]	0.000	0.003	0.884
	6 hr [24]	0.007	0.003	0.035
	24 hr [2,6]	0.030	0.003	<0.001
SAP	2 hr [24]	0.017	0.034	0.622
	6 hr [24]	0.005	0.034	0.879
	24 hr [2,6]	0.143	0.034	<0.001
IL-6	2 hr [6]	0.019	0.178	0.914
	6 hr [2,24]	0.967	0.178	<0.001
	24 hr [6]	0.146	0.178	0.420
MPX	2 hr [24]	-0.020	0.026	0.462
	6 hr [24]	0.034	0.026	0.211
	24 hr [2,6]	0.184	0.026	<0.001
IL-1α	2 hr [24]	0.521	0.840	0.541
	6 hr [24]	-0.884	0.840	0.305
	24 hr [2,6]	-4.452	0.840	<0.001

[2] Indicates the mean is significantly different from the mean at 2 hours
[6] Indicates the mean is significantly different from the mean at 6 hours
[24] Indicates the mean is significantly different from the mean at 24 hours

APPENDIX A

Listings of Analysis Datasets

Task 41 Mod I **Tissue Weight** SAS dataset created by SAS Access from Microsoft Excel file MPX Data.xls

Obs	Date	Animal ID	Site	Exposure	Time Post-Exposure	Tissue Weight
1	13934	22498-1	A	HD	6 hr	0.1091
2	13934	22498-1	B	Sham	6 hr	0.0753
3	13934	22498-1	C	Sham	6 hr	0.0773
4	13934	22498-1	D	HD	6 hr	0.0781
5	13934	22498-2	A	HD	6 hr	0.0859
6	13934	22498-2	B	Sham	6 hr	0.0792
7	13934	22498-2	C	Sham	6 hr	0.0778
8	13934	22498-2	D	HD	6 hr	0.0750
9	13934	22498-6	A	HD	2 hr	0.0939
10	13934	22498-6	B	Sham	2 hr	0.0947
11	13934	22498-6	C	Sham	2 hr	0.0933
12	13934	22498-6	D	HD	2 hr	0.0920
13	13934	22498-7	A	HD	2 hr	0.0930
14	13934	22498-7	B	Sham	2 hr	0.1059
15	13934	22498-7	C	Sham	2 hr	0.0960
16	13934	22498-7	D	HD	2 hr	0.1130
17	13934	22498-11	A	Sham	24 hr	0.1099
18	13934	22498-11	B	HD	24 hr	0.1444
19	13934	22498-11	C	HD	24 hr	0.1044
20	13934	22498-11	D	Sham	24 hr	0.0960
21	13934	22498-12	A	Sham	24 hr	0.0825
22	13934	22498-12	B	HD	24 hr	0.1318
23	13934	22498-12	C	HD	24 hr	0.1118
24	13934	22498-12	D	Sham	24 hr	0.1100
25	13936	22698-1	A	HD	6 hr	0.1145
26	13936	22698-1	B	Sham	6 hr	0.0993
27	13936	22698-1	C	Sham	6 hr	0.1048
28	13936	22698-1	D	HD	6 hr	0.0989
29	13936	22698-16	A	HD	6 hr	0.0941
30	13936	22698-16	B	Sham	6 hr	0.0779
31	13936	22698-16	C	Sham	6 hr	0.0891
32	13936	22698-16	D	HD	6 hr	0.0881
33	13936	22698-6	A	HD	2 hr	0.0963
34	13936	22698-6	B	Sham	2 hr	0.1056
35	13936	22698-6	C	Sham	2 hr	0.0886
36	13936	22698-6	D	HD	2 hr	0.1118
37	13936	22698-7	A	HD	2 hr	0.0878
38	13936	22698-7	B	Sham	2 hr	0.0883
39	13936	22698-7	C	Sham	2 hr	0.0736
40	13936	22698-7	D	HD	2 hr	0.0816
41	13936	22698-11	A	Sham	24 hr	0.0860
42	13936	22698-11	B	HD	24 hr	0.1292
43	13936	22698-11	C	HD	24 hr	0.1138
44	13936	22698-11	D	Sham	24 hr	0.0836
45	13936	22698-12	A	Sham	24 hr	0.0735
46	13936	22698-12	B	HD	24 hr	0.1402
47	13936	22698-12	C	HD	24 hr	0.1009
48	13936	22698-12	D	Sham	24 hr	0.0912
49	13941	30398-1	A	HD	2 hr	0.1281
50	13941	30398-1	B	Sham	2 hr	0.1062
51	13941	30398-1	C	Sham	2 hr	0.0985
52	13941	30398-1	D	HD	2 hr	0.0876
53	13941	30398-2	A	HD	2 hr	0.1022
54	13941	30398-2	B	Sham	2 hr	0.0971
55	13941	30398-2	C	Sham	2 hr	0.1165
56	13941	30398-2	D	HD	2 hr	0.0857
57	13941	30398-3	A	HD	2 hr	0.1032
58	13941	30398-3	B	Sham	2 hr	0.0999
59	13941	30398-3	C	Sham	2 hr	0.0948
60	13941	30398-3	D	HD	2 hr	0.0967

Task 41 Mod I **Tissue Weight** SAS dataset created by SAS Access from Microsoft Excel file MPX Data.xls

Obs	Date	Animal ID	Site	Exposure	Time Post-Exposure	Tissue Weight
61	13941	30398-4	A	HD	2 hr	0.0979
62	13941	30398-4	B	Sham	2 hr	0.1024
63	13941	30398-4	C	Sham	2 hr	0.0881
64	13941	30398-4	D	HD	2 hr	0.0859
65	13941	30398-7	A	HD	6 hr	0.1358
66	13941	30398-7	B	Sham	6 hr	0.1307
67	13941	30398-7	C	Sham	6 hr	0.1145
68	13941	30398-7	D	HD	6 hr	0.0948
69	13941	30398-8	A	HD	6 hr	0.1059
70	13941	30398-8	B	Sham	6 hr	0.0771
71	13941	30398-8	C	Sham	6 hr	0.0666
72	13941	30398-8	D	HD	6 hr	0.0793
73	13941	30398-9	A	HD	6 hr	0.0854
74	13941	30398-9	B	Sham	6 hr	0.0714
75	13941	30398-9	C	Sham	6 hr	0.0820
76	13941	30398-9	D	HD	6 hr	0.0777
77	13941	30398-10	A	Sham	6 hr	0.1069
78	13941	30398-10	B	HD	6 hr	0.1069
79	13941	30398-10	C	HD	6 hr	0.1026
80	13941	30398-10	D	Sham	6 hr	0.0918
81	13941	30398-13	A	Sham	24 hr	0.1059
82	13941	30398-13	B	HD	24 hr	0.1420
83	13941	30398-13	C	HD	24 hr	0.0959
84	13941	30398-13	D	Sham	24 hr	0.0899
85	13941	30398-14	A	Sham	24 hr	0.0892
86	13941	30398-14	B	HD	24 hr	0.1166
87	13941	30398-14	C	HD	24 hr	0.1071
88	13941	30398-14	D	Sham	24 hr	0.1012
89	13941	30398-19	A	Sham	24 hr	0.0938
90	13941	30398-19	B	HD	24 hr	0.1528
91	13941	30398-19	C	HD	24 hr	0.1096
92	13941	30398-19	D	Sham	24 hr	0.0902
93	13941	30398-16	A	Sham	24 hr	0.0861
94	13941	30398-16	B	HD	24 hr	0.1535
95	13941	30398-16	C	HD	24 hr	0.1028
96	13941	30398-16	D	Sham	24 hr	0.0814

Task 41 Module I **SAP** SAS dataset created by SAS Access from Microsoft Excel file SAP Data.xls

Obs	Date	Animal ID	Site	Exposure	Time Post-Exposure	SAP
1	13934	22498-1	A	HD	6 hr	0.13107
2	13934	22498-1	B	Sham	6 hr	0.12139
3	13934	22498-1	C	Sham	6 hr	0.16855
4	13934	22498-1	D	HD	6 hr	0.13972
5	13934	22498-2	A	HD	6 hr	0.14438
6	13934	22498-2	B	Sham	6 hr	0.08205
7	13934	22498-2	C	Sham	6 hr	0.10734
8	13934	22498-2	D	HD	6 hr	0.10088
9	13934	22498-6	A	HD	2 hr	0.24060
10	13934	22498-6	B	Sham	2 hr	0.13027
11	13934	22498-6	C	Sham	2 hr	0.26714
12	13934	22498-6	D	HD	2 hr	0.23405
13	13934	22498-7	A	HD	2 hr	0.01214
14	13934	22498-7	B	Sham	2 hr	0.01668
15	13934	22498-7	C	Sham	2 hr	0.01198
16	13934	22498-7	D	HD	2 hr	0.02415
17	13934	22498-11	A	Sham	24 hr	0.11345
18	13934	22498-11	B	HD	24 hr	0.12029
19	13934	22498-11	C	HD	24 hr	0.23106
20	13934	22498-11	D	Sham	24 hr	0.28179
21	13934	22498-12	A	Sham	24 hr	0.09892
22	13934	22498-12	B	HD	24 hr	0.26985
23	13934	22498-12	C	HD	24 hr	0.13932
24	13934	22498-12	D	Sham	24 hr	0.10933
25	13936	22698-1	A	HD	6 hr	0.06394
26	13936	22698-1	B	Sham	6 hr	0.07845
27	13936	22698-1	C	Sham	6 hr	0.07517
28	13936	22698-1	D	HD	6 hr	0.12062
29	13936	22698-16	A	HD	6 hr	0.07878
30	13936	22698-16	B	Sham	6 hr	0.07209
31	13936	22698-16	C	Sham	6 hr	0.05587
32	13936	22698-16	D	HD	6 hr	0.04710
33	13936	22698-6	A	HD	2 hr	0.18582
34	13936	22698-6	B	Sham	2 hr	0.09788
35	13936	22698-6	C	Sham	2 hr	0.15980
36	13936	22698-6	D	HD	2 hr	0.22505
37	13936	22698-7	A	HD	2 hr	0.04701
38	13936	22698-7	B	Sham	2 hr	0.06971
39	13936	22698-7	C	Sham	2 hr	0.04997
40	13936	22698-7	D	HD	2 hr	0.05289
41	13936	22698-11	A	Sham	24 hr	0.03221
42	13936	22698-11	B	HD	24 hr	0.03269
43	13936	22698-11	C	HD	24 hr	0.04830
44	13936	22698-11	D	Sham	24 hr	0.01731
45	13936	22698-12	A	Sham	24 hr	0.05802
46	13936	22698-12	B	HD	24 hr	0.09650
47	13936	22698-12	C	HD	24 hr	0.09127
48	13936	22698-12	D	Sham	24 hr	0.06826
49	13941	30398-1	A	HD	2 hr	0.06633
50	13941	30398-1	B	Sham	2 hr	0.07433
51	13941	30398-1	C	Sham	2 hr	0.00674
52	13941	30398-1	D	HD	2 hr	0.01838
53	13941	30398-2	A	HD	2 hr	0.02961
54	13941	30398-2	B	Sham	2 hr	0.00764
55	13941	30398-2	C	Sham	2 hr	0.00574
56	13941	30398-2	D	HD	2 hr	0.03240
57	13941	30398-3	A	HD	2 hr	0.00986
58	13941	30398-3	B	Sham	2 hr	0.00676
59	13941	30398-3	C	Sham	2 hr	0.01858

Task 41 Module I **SAP** SAS dataset created by SAS Access from Microsoft Excel file SAP Data.xls

Obs	Date	Animal ID	Site	Exposure	Time Post-Exposure	SAP
60	13941	30398-3	D	HD	2 hr	0.00752
61	13941	30398-4	A	HD	2 hr	0.05577
62	13941	30398-4	B	Sham	2 hr	0.06235
63	13941	30398-4	C	Sham	2 hr	0.06837
64	13941	30398-4	D	HD	2 hr	0.08866
65	13941	30398-7	A	HD	6 hr	0.01020
66	13941	30398-7	B	Sham	6 hr	0.00979
67	13941	30398-7	C	Sham	6 hr	0.01218
68	13941	30398-7	D	HD	6 hr	0.01486
69	13941	30398-8	A	HD	6 hr	0.08846
70	13941	30398-8	B	Sham	6 hr	0.07557
71	13941	30398-8	C	Sham	6 hr	0.10646
72	13941	30398-8	D	HD	6 hr	0.09982
73	13941	30398-9	A	HD	6 hr	0.07751
74	13941	30398-9	B	Sham	6 hr	0.06671
75	13941	30398-9	C	Sham	6 hr	0.10110
76	13941	30398-9	D	HD	6 hr	0.06780
77	13941	30398-10	A	Sham	6 hr	0.10673
78	13941	30398-10	B	HD	6 hr	0.16264
79	13941	30398-10	C	HD	6 hr	0.11331
80	13941	30398-10	D	Sham	6 hr	0.13669
81	13941	30398-13	A	Sham	24 hr	0.22106
82	13941	30398-13	B	HD	24 hr	0.62472
83	13941	30398-13	C	HD	24 hr	0.60363
84	13941	30398-13	D	Sham	24 hr	0.22861
85	13941	30398-14	A	Sham	24 hr	0.08418
86	13941	30398-14	B	HD	24 hr	0.16739
87	13941	30398-14	C	HD	24 hr	0.15603
88	13941	30398-14	D	Sham	24 hr	0.05999
89	13941	30398-19	A	Sham	24 hr	0.41490
90	13941	30398-19	B	HD	24 hr	0.95122
91	13941	30398-19	C	HD	24 hr	0.99420
92	13941	30398-19	D	Sham	24 hr	0.71096
93	13941	30398-16	A	Sham	24 hr	0.09834
94	13941	30398-16	B	HD	24 hr	0.24986
95	13941	30398-16	C	HD	24 hr	0.21640
96	13941	30398-16	D	Sham	24 hr	0.11402

Task 41 Module I **IL-6** SAS dataset created by SAS Access from Microsoft Excel file New IL-6 corrected.xls

Obs	Date	Animal ID	Site	Exposure	Time Post-Exposure	IL-6
1	13934	22498-6	A	HD	2 hr	0.12950
2	13934	22498-7	A	HD	2 hr	0.03732
3	13936	22698-6	A	HD	2 hr	0.04445
4	13936	22698-7	A	HD	2 hr	0.00304
5	13941	30398-1	A	HD	2 hr	0.03348
6	13941	30398-2	A	HD	2 hr	0.14602
7	13941	30398-3	A	HD	2 hr	0.03428
8	13941	30398-4	A	HD	2 hr	-0.00358
9	13934	22498-6	D	HD	2 hr	0.06721
10	13934	22498-7	D	HD	2 hr	0.14884
11	13936	22698-6	D	HD	2 hr	0.07085
12	13936	22698-7	D	HD	2 hr	-0.01614
13	13941	30398-1	D	HD	2 hr	0.01347
14	13941	30398-2	D	HD	2 hr	0.09899
15	13941	30398-3	D	HD	2 hr	-0.00093
16	13941	30398-4	D	HD	2 hr	-0.02452
17	13934	22498-6	B	Sham	2 hr	0.07580
18	13934	22498-7	B	Sham	2 hr	0.07941
19	13936	22698-6	B	Sham	2 hr	0.01427
20	13936	22698-7	B	Sham	2 hr	0.00045
21	13941	30398-1	B	Sham	2 hr	0.01818
22	13941	30398-2	B	Sham	2 hr	0.07996
23	13941	30398-3	B	Sham	2 hr	0.01178
24	13941	30398-4	B	Sham	2 hr	0.01812
25	13934	22498-6	C	Sham	2 hr	0.08774
26	13934	22498-7	C	Sham	2 hr	0.03816
27	13936	22698-6	C	Sham	2 hr	0.03173
28	13936	22698-7	C	Sham	2 hr	-0.02030
29	13941	30398-1	C	Sham	2 hr	0.02464
30	13941	30398-2	C	Sham	2 hr	0.02502
31	13941	30398-3	C	Sham	2 hr	-0.01840
32	13941	30398-4	C	Sham	2 hr	0.00567
33	13934	22498-1	A	HD	6 hr	0.51144
34	13934	22498-2	A	HD	6 hr	1.14072
35	13936	22698-1	A	HD	6 hr	1.12121
36	13936	22698-16	A	HD	6 hr	0.92895
37	13941	30398-7	A	HD	6 hr	0.50961
38	13941	30398-8	A	HD	6 hr	0.75580
39	13941	30398-9	A	HD	6 hr	1.80848
40	13941	30398-10	B	HD	6 hr	3.02182
41	13934	22498-1	D	HD	6 hr	1.01323
42	13934	22498-2	D	HD	6 hr	0.10373
43	13936	22698-1	D	HD	6 hr	1.78655
44	13936	22698-16	D	HD	6 hr	0.30799
45	13941	30398-7	D	HD	6 hr	0.51999
46	13941	30398-8	D	HD	6 hr	0.98152
47	13941	30398-9	D	HD	6 hr	0.53615
48	13941	30398-10	C	HD	6 hr	2.55309
49	13934	22498-1	B	Sham	6 hr	0.27189
50	13934	22498-2	B	Sham	6 hr	0.10015
51	13936	22698-1	B	Sham	6 hr	0.15023
52	13936	22698-16	B	Sham	6 hr	0.00055
53	13941	30398-7	B	Sham	6 hr	0.01306
54	13941	30398-8	B	Sham	6 hr	0.01879
55	13941	30398-9	B	Sham	6 hr	0.03440
56	13941	30398-10	A	Sham	6 hr	0.01273
57	13934	22498-1	C	Sham	6 hr	0.16733
58	13934	22498-2	C	Sham	6 hr	1.32491
59	13936	22698-1	C	Sham	6 hr	0.06812

Task 41 Module I **IL-6** SAS dataset created by SAS Access from Microsoft Excel file New IL-6 corrected.xls

Obs	Date	Animal ID	Site	Exposure	Time Post-Exposure	IL-6
60	13936	22698-16	C	Sham	6 hr	-0.01660
61	13941	30398-7	C	Sham	6 hr	0.03820
62	13941	30398-8	C	Sham	6 hr	0.00395
63	13941	30398-9	C	Sham	6 hr	-0.00209
64	13941	30398-10	D	Sham	6 hr	-0.06434
65	13934	22498-11	B	HD	24 hr	0.10759
66	13934	22498-12	B	HD	24 hr	0.20627
67	13936	22698-11	B	HD	24 hr	0.07299
68	13936	22698-12	B	HD	24 hr	0.07297
69	13941	30398-13	B	HD	24 hr	0.26629
70	13941	30398-14	B	HD	24 hr	0.23654
71	13941	30398-19	B	HD	24 hr	0.24133
72	13941	30398-16	B	HD	24 hr	0.29812
73	13934	22498-11	C	HD	24 hr	0.17820
74	13934	22498-12	C	HD	24 hr	0.11121
75	13936	22698-11	C	HD	24 hr	0.11204
76	13936	22698-12	C	HD	24 hr	0.07350
77	13941	30398-13	C	HD	24 hr	0.26358
78	13941	30398-14	C	HD	24 hr	0.22383
79	13941	30398-19	C	HD	24 hr	0.33689
80	13941	30398-16	C	HD	24 hr	0.30420
81	13934	22498-11	A	Sham	24 hr	0.10923
82	13934	22498-12	A	Sham	24 hr	0.08159
83	13936	22698-11	A	Sham	24 hr	-0.01248
84	13936	22698-12	A	Sham	24 hr	0.04453
85	13941	30398-13	A	Sham	24 hr	0.01738
86	13941	30398-14	A	Sham	24 hr	0.01405
87	13941	30398-19	A	Sham	24 hr	0.11383
88	13941	30398-16	A	Sham	24 hr	0.11431
89	13934	22498-11	D	Sham	24 hr	0.09901
90	13934	22498-12	D	Sham	24 hr	0.06423
91	13936	22698-11	D	Sham	24 hr	0.04649
92	13936	22698-12	D	Sham	24 hr	-0.02023
93	13941	30398-13	D	Sham	24 hr	-0.02169
94	13941	30398-14	D	Sham	24 hr	0.00458
95	13941	30398-19	D	Sham	24 hr	0.09472
96	13941	30398-16	D	Sham	24 hr	0.01991

Task 41 Module I **MPX** SAS dataset created by SAS Access from Microsoft Excel file MPX Data.xls

Obs	Date	Animal ID	Site	Exposure	Time Post-Exposure	MPX
1	13934	22498-1	A	HD	6 hr	0.16022
2	13934	22498-1	B	Sham	6 hr	0.17142
3	13934	22498-1	C	Sham	6 hr	0.11353
4	13934	22498-1	D	HD	6 hr	0.19135
5	13934	22498-2	A	HD	6 hr	0.45389
6	13934	22498-2	B	Sham	6 hr	0.26585
7	13934	22498-2	C	Sham	6 hr	0.19643
8	13934	22498-2	D	HD	6 hr	0.12601
9	13934	22498-6	A	HD	2 hr	0.26823
10	13934	22498-6	B	Sham	2 hr	0.34255
11	13934	22498-6	C	Sham	2 hr	0.13977
12	13934	22498-6	D	HD	2 hr	0.11132
13	13934	22498-7	A	HD	2 hr	0.11375
14	13934	22498-7	B	Sham	2 hr	0.07277
15	13934	22498-7	C	Sham	2 hr	0.03464
16	13934	22498-7	D	HD	2 hr	0.01478
17	13934	22498-11	A	Sham	24 hr	0.20145
18	13934	22498-11	B	HD	24 hr	0.41318
19	13934	22498-11	C	HD	24 hr	0.24518
20	13934	22498-11	D	Sham	24 hr	0.07219
21	13934	22498-12	A	Sham	24 hr	0.19412
22	13934	22498-12	B	HD	24 hr	0.56696
23	13934	22498-12	C	HD	24 hr	0.36709
24	13934	22498-12	D	Sham	24 hr	0.07092
25	13936	22698-1	A	HD	6 hr	0.22868
26	13936	22698-1	B	Sham	6 hr	0.10406
27	13936	22698-1	C	Sham	6 hr	0.08668
28	13936	22698-1	D	HD	6 hr	0.09008
29	13936	22698-16	A	HD	6 hr	0.20672
30	13936	22698-16	B	Sham	6 hr	0.15010
31	13936	22698-16	C	Sham	6 hr	0.03305
32	13936	22698-16	D	HD	6 hr	0.07501
33	13936	22698-6	A	HD	2 hr	0.19292
34	13936	22698-6	B	Sham	2 hr	0.13875
35	13936	22698-6	C	Sham	2 hr	0.05292
36	13936	22698-6	D	HD	2 hr	0.01230
37	13936	22698-7	A	HD	2 hr	0.04907
38	13936	22698-7	B	Sham	2 hr	0.08988
39	13936	22698-7	C	Sham	2 hr	0.04877
40	13936	22698-7	D	HD	2 hr	0.01741
41	13936	22698-11	A	Sham	24 hr	0.16202
42	13936	22698-11	B	HD	24 hr	0.35387
43	13936	22698-11	C	HD	24 hr	0.06463
44	13936	22698-11	D	Sham	24 hr	0.02208
45	13936	22698-12	A	Sham	24 hr	0.16686
46	13936	22698-12	B	HD	24 hr	0.24977
47	13936	22698-12	C	HD	24 hr	0.16104
48	13936	22698-12	D	Sham	24 hr	0.02942
49	13941	30398-1	A	HD	2 hr	0.12650
50	13941	30398-1	B	Sham	2 hr	0.22461
51	13941	30398-1	C	Sham	2 hr	0.09936
52	13941	30398-1	D	HD	2 hr	0.05018
53	13941	30398-2	A	HD	2 hr	0.21620
54	13941	30398-2	B	Sham	2 hr	0.27690
55	13941	30398-2	C	Sham	2 hr	0.03119
56	13941	30398-2	D	HD	2 hr	0.08223
57	13941	30398-3	A	HD	2 hr	0.22333
58	13941	30398-3	B	Sham	2 hr	0.24068
59	13941	30398-3	C	Sham	2 hr	0.13617
60	13941	30398-3	D	HD	2 hr	0.08288

Task 41 Module I **MPX** SAS dataset created by SAS Access from Microsoft Excel file MPX Data.xls

Obs	Date	Animal ID	Site	Exposure	Time Post-Exposure	MPX
61	13941	30398-4	A	HD	2 hr	0.29215
62	13941	30398-4	B	Sham	2 hr	0.24368
63	13941	30398-4	C	Sham	2 hr	0.04308
64	13941	30398-4	D	HD	2 hr	0.04521
65	13941	30398-7	A	HD	6 hr	0.30092
66	13941	30398-7	B	Sham	6 hr	0.16883
67	13941	30398-7	C	Sham	6 hr	0.05989
68	13941	30398-7	D	HD	6 hr	0.07605
69	13941	30398-8	A	HD	6 hr	0.15510
70	13941	30398-8	B	Sham	6 hr	0.16352
71	13941	30398-8	C	Sham	6 hr	0.12892
72	13941	30398-8	D	HD	6 hr	0.11431
73	13941	30398-9	A	HD	6 hr	0.30299
74	13941	30398-9	B	Sham	6 hr	0.11663
75	13941	30398-9	C	Sham	6 hr	0.05254
76	13941	30398-9	D	HD	6 hr	0.10466
77	13941	30398-10	A	Sham	6 hr	0.67558
78	13941	30398-10	B	HD	6 hr	0.22573
79	13941	30398-10	C	HD	6 hr	0.28533
80	13941	30398-10	D	Sham	6 hr	0.06352
81	13941	30398-13	A	Sham	24 hr	0.40815
82	13941	30398-13	B	HD	24 hr	0.51777
83	13941	30398-13	C	HD	24 hr	0.17952
84	13941	30398-13	D	Sham	24 hr	0.01939
85	13941	30398-14	A	Sham	24 hr	0.18791
86	13941	30398-14	B	HD	24 hr	0.26263
87	13941	30398-14	C	HD	24 hr	0.17114
88	13941	30398-14	D	Sham	24 hr	0.04184
89	13941	30398-19	A	Sham	24 hr	0.33740
90	13941	30398-19	B	HD	24 hr	0.49105
91	13941	30398-19	C	HD	24 hr	0.18678
92	13941	30398-19	D	Sham	24 hr	0.08057
93	13941	30398-16	A	Sham	24 hr	0.37796
94	13941	30398-16	B	HD	24 hr	0.64374
95	13941	30398-16	C	HD	24 hr	0.54888
96	13941	30398-16	D	Sham	24 hr	0.10206

Task 41 Module I **IL-1α** SAS dataset created by SAS Access from Microsoft Excel file IL-1 Data.xls

Obs	Date	Animal ID	Site	Exposure	Time Post-Exposure	IL-1 alpha
1	13934	22498-1	A	HD	6 hr	5.3296
2	13934	22498-1	B	Sham	6 hr	10.6268
3	13934	22498-1	C	Sham	6 hr	15.8361
4	13934	22498-1	D	HD	6 hr	12.8155
5	13934	22498-2	A	HD	6 hr	8.7098
6	13934	22498-2	B	Sham	6 hr	8.7885
7	13934	22498-2	C	Sham	6 hr	9.2971
8	13934	22498-2	D	HD	6 hr	10.1302
9	13934	22498-6	A	HD	2 hr	10.9124
10	13934	22498-6	B	Sham	2 hr	6.7913
11	13934	22498-6	C	Sham	2 hr	7.9574
12	13934	22498-6	D	HD	2 hr	9.3542
13	13934	22498-7	A	HD	2 hr	6.4684
14	13934	22498-7	B	Sham	2 hr	9.0074
15	13934	22498-7	C	Sham	2 hr	6.2704
16	13934	22498-7	D	HD	2 hr	12.7381
17	13934	22498-11	A	Sham	24 hr	8.5679
18	13934	22498-11	B	HD	24 hr	2.2173
19	13934	22498-11	C	HD	24 hr	6.0810
20	13934	22498-11	D	Sham	24 hr	7.7809
21	13934	22498-12	A	Sham	24 hr	5.1393
22	13934	22498-12	B	HD	24 hr	2.5819
23	13934	22498-12	C	HD	24 hr	2.6033
24	13934	22498-12	D	Sham	24 hr	4.6040
25	13936	22698-1	A	HD	6 hr	6.9128
26	13936	22698-1	B	Sham	6 hr	5.9513
27	13936	22698-1	C	Sham	6 hr	9.3876
28	13936	22698-1	D	HD	6 hr	10.5084
29	13936	22698-16	A	HD	6 hr	9.4732
30	13936	22698-16	B	Sham	6 hr	9.1080
31	13936	22698-16	C	Sham	6 hr	8.3423
32	13936	22698-16	D	HD	6 hr	7.3006
33	13936	22698-6	A	HD	2 hr	6.6872
34	13936	22698-6	B	Sham	2 hr	3.9835
35	13936	22698-6	C	Sham	2 hr	7.0914
36	13936	22698-6	D	HD	2 hr	5.3208
37	13936	22698-7	A	HD	2 hr	5.0005
38	13936	22698-7	B	Sham	2 hr	8.4911
39	13936	22698-7	C	Sham	2 hr	5.3374
40	13936	22698-7	D	HD	2 hr	5.0860
41	13936	22698-11	A	Sham	24 hr	8.5599
42	13936	22698-11	B	HD	24 hr	1.9918
43	13936	22698-11	C	HD	24 hr	4.5335
44	13936	22698-11	D	Sham	24 hr	6.5975
45	13936	22698-12	A	Sham	24 hr	7.9756
46	13936	22698-12	B	HD	24 hr	2.1293
47	13936	22698-12	C	HD	24 hr	3.4450
48	13936	22698-12	D	Sham	24 hr	10.7345
49	13941	30398-1	A	HD	2 hr	3.0362
50	13941	30398-1	B	Sham	2 hr	5.0785
51	13941	30398-1	C	Sham	2 hr	6.5224
52	13941	30398-1	D	HD	2 hr	6.5280
53	13941	30398-2	A	HD	2 hr	7.1724
54	13941	30398-2	B	Sham	2 hr	6.6708
55	13941	30398-2	C	Sham	2 hr	6.6869
56	13941	30398-2	D	HD	2 hr	10.3798
57	13941	30398-3	A	HD	2 hr	6.2026
58	13941	30398-3	B	Sham	2 hr	7.3498
59	13941	30398-3	C	Sham	2 hr	6.1337

Task 41 Module I **IL-1α** SAS dataset created by SAS Access from Microsoft Excel file IL-1 Data.xls

Obs	Date	Animal ID	Site	Exposure	Time Post-Exposure	IL-1 alpha
60	13941	30398-3	D	HD	2 hr	6.3854
61	13941	30398-4	A	HD	2 hr	7.3488
62	13941	30398-4	B	Sham	2 hr	6.3668
63	13941	30398-4	C	Sham	2 hr	11.6026
64	13941	30398-4	D	HD	2 hr	11.0644
65	13941	30398-7	A	HD	6 hr	2.1844
66	13941	30398-7	B	Sham	6 hr	2.5906
67	13941	30398-7	C	Sham	6 hr	4.0297
68	13941	30398-7	D	HD	6 hr	5.0111
69	13941	30398-8	A	HD	6 hr	3.8101
70	13941	30398-8	B	Sham	6 hr	8.4620
71	13941	30398-8	C	Sham	6 hr	10.8430
72	13941	30398-8	D	HD	6 hr	8.5394
73	13941	30398-9	A	HD	6 hr	14.2752
74	13941	30398-9	B	Sham	6 hr	6.9179
75	13941	30398-9	C	Sham	6 hr	12.8296
76	13941	30398-9	D	HD	6 hr	9.5719
77	13941	30398-10	A	Sham	6 hr	5.2956
78	13941	30398-10	B	HD	6 hr	6.0056
79	13941	30398-10	C	HD	6 hr	4.9443
80	13941	30398-10	D	Sham	6 hr	11.3575
81	13941	30398-13	A	Sham	24 hr	3.0940
82	13941	30398-13	B	HD	24 hr	1.9396
83	13941	30398-13	C	HD	24 hr	7.3824
84	13941	30398-13	D	Sham	24 hr	6.1021
85	13941	30398-14	A	Sham	24 hr	5.9316
86	13941	30398-14	B	HD	24 hr	4.1192
87	13941	30398-14	C	HD	24 hr	4.7445
88	13941	30398-14	D	Sham	24 hr	12.4248
89	13941	30398-19	A	Sham	24 hr	5.0998
90	13941	30398-19	B	HD	24 hr	2.0404
91	13941	30398-19	C	HD	24 hr	6.3301
92	13941	30398-19	D	Sham	24 hr	10.4670
93	13941	30398-16	A	Sham	24 hr	13.0581
94	13941	30398-16	B	HD	24 hr	1.9687
95	13941	30398-16	C	HD	24 hr	10.9882
96	13941	30398-16	D	Sham	24 hr	20.1835

APPENDIX H

Module II Statistical Analyses

Battelle
. . . Putting Technology To Work

Project Number G1555-B41ASTAT (3104)

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Date August 3, 2000

To **Carol Sabourin**

From Nancy Niemuth

Subject **Statistical Analysis of Study G1555-41A, MREF Task 95-41 - Module II - Revision 2**

The attached report describes the statistical analysis of tissue weight, SAP, IL-6, and MPX data collected under Module II of Task 95-41. In preparing supporting materials for the QA review of this report, we found that Model 2 results had been reported for tissue weights in Table 5 rather than Model 3 results. This report corrects the tissue weight results, presented in Table 5. No other changes were made. The study conclusions were not affected by this change. An electronic copy of the statistical report will be provided for use in preparing the final report on this study.

Please call me at (614) 424-3231 if you have any questions.

NAN:llj
Attachment

For Review and Approval

	Name	Initials	Date
Originator	Nancy Niemuth	N	8/3/00
Concurrence	Brandon Wood	BJW	8/3/00
Approved	Bill Rosebrough	WRR	8/4/00

Sent: Interoffice mail

Statistical Report for MREF Task 95-41 Module II Data

August 3, 2000

Introduction

Experiments were conducted under MREF Task 95-41 Module II to determine the efficacy of two drug treatments, ICD 2723 (Olvanil or OLV) and ICD 2845 (Dexamethasone or DEX), in moderating the effect of HD exposure in the euthymic hairless mouse model. Four exposure sites were available on the back of each animal. Sites A and D were treated (OLV, DEX, or none) and exposed to HD vapor for 6 minutes; Sites B and C were sham sites. There were 10 animals in each drug treatment group and 8 control animals that received no drug treatment. The average response of the two drug treated/HD-exposed sites or two sham sites within each animal was used as the endpoint for statistical analysis. The endpoints evaluated were tissue weight, SAP, IL-6, and MPX.

Methods

Data from several animals were not included in the statistical analysis for Module II. Data from the following three animals were removed from both the SAP and IL-6 analysis for lack of a protein assay: 01/07/99-15, 01/11/99-11, and 01/11/99-12. The SAP endpoint for animal 01/11/99-6 was recalculated because one of the replicate measures was out of the range of the assay. Data from animal 01/11/99-05 were removed from the SAP analysis as all three replicate measurements were out of the range. In addition, the IL-6 data included several negative responses ranging from –0.01 to –0.20. To avoid introducing bias to the statistical analysis, these values were not changed, although it is recognized that IL-6 levels cannot fall below zero.

A two-stage approach was used to analyze each endpoint, which entailed fitting analysis of variance (ANOVA) models to appropriate subsets of the data. The first model (Model 1) was fitted to data from the sham sites. Model 1 tested for a systemic effect due to drug treatment, for each drug treatment compound compared to the control. Based on the results of Model 1 analysis, either Model 2 or Model 3 was selected for the second stage.

If no significant differences were found in Model 1 (indicating there was no systemic effect due to either drug treatment compound) then Model 2 was implemented. Model 2 was fitted to data from the drug treated/HD-exposed sites only. Model 2 tested for differences between HD-exposed sites on drug treated and control animals.

If significant differences were found between sham site responses for either drug treatment compound compared to control, indicating a systemic effect due to a drug treatment compound, Model 3 was employed. Model 3 tested for differences between treatment and control animals after adjusting for differences in sham site responses. For each animal, the

response variable for Model 3 was calculated as the difference between the drug treated/ HD-exposed site mean and sham site mean.

The ANOVA Models 1, 2, and 3 fitted to the tissue weight, SAP, IL-6, and MPX Module II data took the following form:

$$Y_{ij} = \mu + \beta_i + \varepsilon_{ij},$$

where Y_{ij} = tissue weight, SAP, IL-6, or MPX response for j^{th} animal receiving i^{th} treatment
μ = overall average value of the response
β_i = effect of i^{th} treatment
ε = uncontrolled variation.

The ANOVA models were fitted using the SAS (V8) MIXED procedure. For each endpoint, Dunnett's multiple comparison procedure was used to compare each drug treatment group to control. These comparisons are summarized for each model, as follows:

- Model 1: $SHAM_{trt} - SHAM_{ctl}$
- Model 2: $HD_{trt} - HD_{ctl}$
- Model 3: $(HD_{trt} - SHAM_{trt}) - (HD_{ctl} - SHAM_{ctl})$

where SHAM and HD are the means for sham sites and drug treated/HD-exposed sites, respectively, for the treatment (trt) or control (ctl) group indicated by the subscript. In addition, the relative response for each group and percent reduction in response for drug treated groups compared to control were calculated as

$$\text{Relative Response} = 100 * (HD - SHAM)/ SHAM$$

$$\text{Percent Change} = \frac{100 * [(HD_{trt} - SHAM_{trt})/ SHAM_{trt} - (HD_{ctl} - SHAM_{ctl})/ SHAM_{ctl}]}{[(HD_{ctl} - SHAM_{ctl})/SHAM_{ctl}]}$$

Results

Descriptive statistics for tissue weight, SAP, IL-6, and MPX for each response endpoint are displayed in Tables 1 through 4, respectively. Average SAP values appear to be somewhat lower in the control animals than in those drug treated with either DEX or OLV. Average values were clearly greater for control animals than those receiving drug treatments for tissue weight, IL-6, and MPX.

A summary of the relative response and percent change in response relative to control for all endpoints is provided in Table 5. Parameter estimates and results for the ANOVA models fitted to the tissue weight, SAP, IL-6, and MPX data are presented in Table 6 and summarized below. Model estimated differences between drug treated/HD-exposed sites and sham sites are presented in Table 7. Listings of the raw data used in the statistical analysis are provided in Appendix A.

A statistically significant increase in tissue weights from 0.094 g to 0.135 g ($p=<0.001$) was associated with HD exposure in the Control group (Tables 1 and 7). Model 1 comparisons of sham sites indicated that there was a systemic effect due to drug treatment for the tissue weight endpoint. Thus, Model 3 was fitted to examine the effects of drug treatment on HD-exposed sites after adjusting for differences in sham site responses. This model indicated that tissue weight increases for both drug treatment compounds were statistically significantly less than tissue weight increases in control animals. Note that, although tissue weight increases were reduced in drug treated groups compared to controls, tissue weights did statistically significantly increase compared to sham sites (Tables 1 and 7).

The effect of HD-exposure on SAP levels was not statistically significant (Tables 2 and 7). Model 1 comparisons of sham sites indicated that there was no systemic effect due to drug treatment. Thus, Model 2 was fitted to examine the effects of drug treatments directly on HD-exposed sites. This model indicated that the drug treatments did not have a statistically significant effect on SAP levels in the HD-exposed sites. A statistically significant increase in SAP values between HD-exposed and sham sites was found for DEX (Table 7).

For IL-6, a statistically significant increase from –0.043 pg/mg/mg to 0.616 pg/mg/mg ($p=<0.001$) was associated with HD-exposure in the Control group (Tables 3 and 7). Model 1 comparisons of sham sites indicated there was no systemic effect due to drug treatment. Model 2 was fitted to examine the effects of drug treatments directly on HD-exposed sites. This model indicated that the IL-6 levels in drug treated/HD-exposed sites were statistically significantly less than IL-6 levels in HD-exposed sites on control animals. The effect of HD-exposure on IL-6 levels was not statistically significant for either of the drug treatments (Table 7).

A statistically significant increase in MPX levels from 0.464 U/mg to 0.802 U/mg was associated with HD exposure in the Control group animals (Tables 4 and 7). Model 1 comparisons of sham sites indicated significant differences among sham sites. Model 3 was fitted to examine the effects of drug treatments on HD-exposed sites after adjusting for differences in sham site responses. This model indicated that both drug treatment compounds resulted in a statistically significant reduction in HD-induced response compared to control values. Neither drug treatment had a statistically significant effect on MPX values for HD-exposure (Table 7).

Conclusions

Drug treatments were found to have a systemic effect, indicated by statistically significant differences in sham site responses, for tissue weight and MPX endpoints. The statistical analysis accounted for this systemic effect on those parameters and found a significant reduction in the response to HD exposure, after accounting for this effect. Significant decreases in tissue weight, IL-6, and MPX responses were found for both drug treatments as compared to the untreated animals. The drug treatments appeared to increase the SAP values slightly, but the effect was not statistically significant.

Table 1. Descriptive Statistics for Tissue Weight for Each Drug Treatment Group

Group	Treatment	Exposure	Number of Animals	Tissue Weight (g)			
				Mean	SD	Relative Response[1]	Percent Change[2]
Control	None	Sham	8	0.094	0.012	44	NA
	None	HD	8	0.135	0.021		
Olvanil	None	Sham	10	0.083	0.007	21	-53
	Olvanil	HD	10	0.100	0.023		
Dexamethasone	None	Sham	10	0.069	0.006	23	-48
	Dexamethasone	HD	10	0.085	0.011		

[1] Relative Response = 100 * (HD – SHAM)/ SHAM.

[2] Percent Change = 100 * $[(HD_{trt} - SHAM_{trt})/ SHAM_{trt} - (HD_{ctl} - SHAM_{ctl})/ SHAM_{ctl}]/[(HD_{ctl} - SHAM_{ctl})/ SHAM_{ctl}]$.

Table 2. Descriptive Statistics for SAP for Each Drug Treatment Group

Group	Treatment	Exposure	Number of Animals[1]	SAP (ng/mg/mg)			
				Mean	SD	Relative Response[2]	Percent Change[3]
Control	None	Sham	5	0.251	0.223	73	NA
	None	HD	5	0.435	0.187		
Olvanil	None	Sham	9	0.515	0.484	23	-68
	Olvanil	HD	9	0.635	0.518		
Dexamethasone	None	Sham	10	0.331	0.164	129	76
	Dexamethasone	HD	10	0.757	0.377		

[1] Three animals without a protein analysis at all sites were removed from the analysis (010799-15, 011199-11, and 011199-12). In addition, animal 011199-5 was removed and SAP was recalculated for animal 011199-6, because the SAP data was out of range of the instrument.

[2] Relative Response = 100 * (HD – SHAM)/ SHAM.

[3] Percent Change = 100 * $[(HD_{trt} - SHAM_{trt})/ SHAM_{trt} - (HD_{ctl} - SHAM_{ctl})/ SHAM_{ctl}]/[(HD_{ctl} - SHAM_{ctl})/ SHAM_{ctl}]$.

Table 3. Descriptive Statistics for IL-6 for Each Drug Treatment Group

Group	Treatment	Exposure	Number of Animals[1]	IL-6 (pg/mg/mg)			
				Mean	SD	Relative Response[2]	Percent Change[3]
Control	None	Sham	5	-0.043[4]	0.016	-1519	NA
	None	HD	5	0.616	0.553		
Olvanil	None	Sham	10	0.115	0.189	108	-107
	Olvanil	HD	10	0.239	0.099		
Dexamethasone	None	Sham	10	0.089	0.192	-15	-99
	Dexamethasone	HD	10	0.075	0.139		

[1] Three animals without a protein analysis at all sites were removed from the IL-6 analysis (010799-15, 011199-11, and 011199-12).

[2] Relative Response = 100 * (HD – SHAM)/ SHAM.

[3] Percent Change = 100 * $[(HD_{trt} - SHAM_{trt})/ SHAM_{trt} - (HD_{ctl} - SHAM_{ctl})/ SHAM_{ctl}]/[(HD_{ctl} - SHAM_{ctl})/ SHAM_{ctl}]$.

[4] The IL-6 data included several negative responses ranging from –0.01 to –0.20. To avoid introducing bias to the statistical analysis, these values were not changed, although it is recognized that IL-6 levels cannot fall below zero.

Table 4. Descriptive Statistics for MPX for Each Drug Treatment Group

Group	Treatment	Exposure	Number of Animals	MPX (U/mg)			
				Mean	SD	Relative Response[1]	Percent Change[2]
Control	None	Sham	8	0.464	0.224	73	NA
	None	HD	8	0.802	0.232		
Olvanil	None	Sham	10	0.423	0.141	17	-76
	Olvanil	HD	10	0.496	0.157		
Dexamethasone	None	Sham	10	0.142	0.069	40	-46
	Dexamethasone	HD	10	0.198	0.090		

[1] Relative Response = 100 * (HD – SHAM)/ SHAM.

[2] Percent Change = 100 * $[(HD_{trt} - SHAM_{trt})/ SHAM_{trt} - (HD_{ctl} - SHAM_{ctl})/ SHAM_{ctl}]/[(HD_{ctl} - SHAM_{ctl})/ SHAM_{ctl}]$.

Table 5. Relative Response and Percent Reduction in Response Relative to Control for Tissue Weight, SAP, IL-6, and MPX Data Collected in Module II

Endpoint	Group	Relative Response[1] (%)	Percent Change Relative to Control[2] (%)
Tissue Weight	Control	44	NA
	Olvanil	21	-53
	Dexamethasone	23	-48
SAP	Control	73	NA
	Olvanil	23	-68
	Dexamethasone	129	76
IL-6	Control	-1519	NA
	Olvanil	108	-107
	Dexamethasone	-15	-99
MPX	Control	73	NA
	Olvanil	17	-76
	Dexamethasone	40	-46

[1] Relative Response = 100 * (HD – SHAM)/ SHAM.

[2] Percent Change = 100 * $[(HD_{trt} - SHAM_{trt})/ SHAM_{trt} - (HD_{ctl} - SHAM_{ctl})/ SHAM_{ctl}]/[(HD_{ctl} - SHAM_{ctl})/ SHAM_{ctl}]$.

Table 6. Model Estimated Differences in Means for Tissue Weight, SAP, IL-6, and MPX

Parameter of Interest	Model 1 Results				Model Chosen	Final Model Results		
	Comparison	Estimated Difference (sham sites)	SE	p-Value		Estimated Difference Due to Drug Treatment	SE	p-Value
Tissue Weight	OLV vs Control	-0.011	0.004	0.025	3	-0.024	0.008	0.010
	DEX vs Control	-0.024	0.004	<0.001		-0.025	0.008	0.007
SAP	OLV vs Control	0.263	0.185	0.268	2	0.199	0.230	0.573
	DEX vs Control	0.079	0.182	0.858		0.322	0.226	0.266
IL-6	OLV vs Control	0.158	0.095	0.175	2	-0.377	0.142	0.026
	DEX vs Control	0.132	0.095	0.276		-0.541	0.142	0.002
MPX	OLV vs Control	-0.042	0.072	0.782	3	-0.264	0.064	<0.001
	DEX vs Control	-0.322	0.072	<0.001		-0.281	0.064	<0.001

Table 7. Model Estimated Differences between HD-Exposed Sites and Sham Sites within Each Treatment Group

Endpoint	Group	Estimated Difference (HD-Sham)	SE	p-Value (T-Test)
Tissue Weight	Control	0.041	0.006	<0.001
	Olvanil	0.017	0.005	0.003
	Dexamethasone	0.016	0.005	0.005
SAP	Control	0.184	0.140	0.204
	Olvanil	0.120	0.105	0.264
	Dexamethasone	0.426	0.099	<0.001
IL-6	Control	0.659	0.119	<0.001
	Olvanil	0.124	0.084	0.156
	Dexamethasone	-0.013	0.054	0.876
MPX	Control	0.337	0.048	<0.001
	Olvanil	0.074	0.043	0.098
	Dexamethasone	0.056	0.043	0.201

APPENDIX A:

LISTINGS OF ANALYSIS DATASETS

Tissue Weight from SAP Module II SAS Dataset Created by DBMS Copy from the Microsoft Excel File KB Module II Summary of SAP.xls - SAP, 010799

Obs	Dose Date	Animal ID	Dose Site	Group	Drug Treatment	Exposure	Tissue Weight
1	01/07/99	1	A	Olvanil	Olvanil	HD	0.0985
2	01/07/99	2	A	Olvanil	Olvanil	HD	0.0978
3	01/07/99	3	A	Olvanil	Olvanil	HD	0.1407
4	01/07/99	4	A	Olvanil	Olvanil	HD	0.1015
5	01/07/99	5	A	Olvanil	Olvanil	HD	0.1501
6	01/07/99	6	A	Dexamethasone	Dexamethasone	HD	0.0872
7	01/07/99	7	A	Dexamethasone	Dexamethasone	HD	0.0882
8	01/07/99	8	A	Dexamethasone	Dexamethasone	HD	0.1183
9	01/07/99	9	A	Dexamethasone	Dexamethasone	HD	0.0934
10	01/07/99	10	A	Dexamethasone	Dexamethasone	HD	0.0828
11	01/07/99	11	A	Control	None	HD	0.1714
12	01/07/99	12	A	Control	None	HD	0.1696
13	01/07/99	13	A	Control	None	HD	0.1402
14	01/07/99	14	A	Control	None	HD	0.1099
15	01/07/99	15	A	Control	None	HD	0.1778
16	01/07/99	1	B	Olvanil	None	Sham	0.1035
17	01/07/99	2	B	Olvanil	None	Sham	0.0919
18	01/07/99	3	B	Olvanil	None	Sham	0.0767
19	01/07/99	4	B	Olvanil	None	Sham	0.1037
20	01/07/99	5	B	Olvanil	None	Sham	0.0850
21	01/07/99	6	B	Dexamethasone	None	Sham	0.0689
22	01/07/99	7	B	Dexamethasone	None	Sham	0.0670
23	01/07/99	8	B	Dexamethasone	None	Sham	0.0660
24	01/07/99	9	B	Dexamethasone	None	Sham	0.0730
25	01/07/99	10	B	Dexamethasone	None	Sham	0.0652
26	01/07/99	11	B	Control	None	Sham	0.1109
27	01/07/99	12	B	Control	None	Sham	0.1150
28	01/07/99	13	B	Control	None	Sham	0.1009
29	01/07/99	14	B	Control	None	Sham	0.0866
30	01/07/99	15	B	Control	None	Sham	0.0953
31	01/07/99	1	C	Olvanil	None	Sham	0.0743
32	01/07/99	2	C	Olvanil	None	Sham	0.0808
33	01/07/99	3	C	Olvanil	None	Sham	0.0936
34	01/07/99	4	C	Olvanil	None	Sham	0.0836
35	01/07/99	5	C	Olvanil	None	Sham	0.0856
36	01/07/99	6	C	Dexamethasone	None	Sham	0.0768
37	01/07/99	7	C	Dexamethasone	None	Sham	0.0651
38	01/07/99	8	C	Dexamethasone	None	Sham	0.0647
39	01/07/99	9	C	Dexamethasone	None	Sham	0.0626
40	01/07/99	10	C	Dexamethasone	None	Sham	0.0755
41	01/07/99	11	C	Control	None	Sham	0.0824
42	01/07/99	12	C	Control	None	Sham	0.1041
43	01/07/99	13	C	Control	None	Sham	0.0948
44	01/07/99	14	C	Control	None	Sham	0.0875
45	01/07/99	15	C	Control	None	Sham	0.0918
46	01/07/99	1	D	Olvanil	Olvanil	HD	0.0936
47	01/07/99	2	D	Olvanil	Olvanil	HD	0.1014
48	01/07/99	3	D	Olvanil	Olvanil	HD	0.1452
49	01/07/99	4	D	Olvanil	Olvanil	HD	0.0956
50	01/07/99	5	D	Olvanil	Olvanil	HD	0.1313
51	01/07/99	6	D	Dexamethasone	Dexamethasone	HD	0.0847
52	01/07/99	7	D	Dexamethasone	Dexamethasone	HD	0.0752
53	01/07/99	8	D	Dexamethasone	Dexamethasone	HD	0.0676
54	01/07/99	9	D	Dexamethasone	Dexamethasone	HD	0.0941
55	01/07/99	10	D	Dexamethasone	Dexamethasone	HD	0.0760
56	01/07/99	11	D	Control	None	HD	0.1480
57	01/07/99	12	D	Control	None	HD	0.1227
58	01/07/99	13	D	Control	None	HD	0.1022
59	01/07/99	14	D	Control	None	HD	0.1069
60	01/07/99	15	D	Control	None	HD	0.1256

Tissue Weight from SAP Module II SAS Dataset Created by DBMS Copy from the Microsoft Excel File KB Module II Summary of SAP.xls - SAP, 011199

Obs	Dose Date	Animal ID	Dose Site	Group	Drug Treatment	Exposure	Tissue Weight
1	01/07/99	1	A	Olvanil	Olvanil	HD	0.0776
2	01/07/99	2	A	Olvanil	Olvanil	HD	0.0732
3	01/07/99	3	A	Olvanil	Olvanil	HD	0.0874
4	01/07/99	4	A	Olvanil	Olvanil	HD	0.1028
5	01/07/99	5	A	Olvanil	Olvanil	HD	0.0891
6	01/07/99	6	A	Dexamethasone	Dexamethasone	HD	0.0665
7	01/07/99	7	A	Dexamethasone	Dexamethasone	HD	0.0924
8	01/07/99	8	A	Dexamethasone	Dexamethasone	HD	0.0843
9	01/07/99	9	A	Dexamethasone	Dexamethasone	HD	0.0819
10	01/07/99	10	A	Dexamethasone	Dexamethasone	HD	0.1100
11	01/07/99	11	A	Control	None	HD	0.1031
12	01/07/99	12	A	Control	None	HD	0.1473
13	01/07/99	13	A	Control	None	HD	0.1540
14	01/07/99	1	B	Olvanil	None	Sham	0.0661
15	01/07/99	2	B	Olvanil	None	Sham	0.0904
16	01/07/99	3	B	Olvanil	None	Sham	0.0773
17	01/07/99	4	B	Olvanil	None	Sham	0.0866
18	01/07/99	5	B	Olvanil	None	Sham	0.0717
19	01/07/99	6	B	Dexamethasone	None	Sham	0.0595
20	01/07/99	7	B	Dexamethasone	None	Sham	0.0731
21	01/07/99	8	B	Dexamethasone	None	Sham	0.0710
22	01/07/99	9	B	Dexamethasone	None	Sham	0.0677
23	01/07/99	10	B	Dexamethasone	None	Sham	0.0751
24	01/07/99	11	B	Control	None	Sham	0.0769
25	01/07/99	12	B	Control	None	Sham	0.1042
26	01/07/99	13	B	Control	None	Sham	0.1093
27	01/07/99	1	C	Olvanil	None	Sham	0.0688
28	01/07/99	2	C	Olvanil	None	Sham	0.0803
29	01/07/99	3	C	Olvanil	None	Sham	0.0790
30	01/07/99	4	C	Olvanil	None	Sham	0.0850
31	01/07/99	5	C	Olvanil	None	Sham	0.0794
32	01/07/99	6	C	Dexamethasone	None	Sham	0.0595
33	01/07/99	7	C	Dexamethasone	None	Sham	0.0657
34	01/07/99	8	C	Dexamethasone	None	Sham	0.0903
35	01/07/99	9	C	Dexamethasone	None	Sham	0.0676
36	01/07/99	10	C	Dexamethasone	None	Sham	0.0730
37	01/07/99	11	C	Control	None	Sham	0.0620
38	01/07/99	12	C	Control	None	Sham	0.0838
39	01/07/99	13	C	Control	None	Sham	0.0958
40	01/07/99	1	D	Olvanil	Olvanil	HD	0.0710
41	01/07/99	2	D	Olvanil	Olvanil	HD	0.0865
42	01/07/99	3	D	Olvanil	Olvanil	HD	0.0798
43	01/07/99	4	D	Olvanil	Olvanil	HD	0.0940
44	01/07/99	5	D	Olvanil	Olvanil	HD	0.0917
45	01/07/99	6	D	Dexamethasone	Dexamethasone	HD	0.0572
46	01/07/99	7	D	Dexamethasone	Dexamethasone	HD	0.0980
47	01/07/99	8	D	Dexamethasone	Dexamethasone	HD	0.0699
48	01/07/99	9	D	Dexamethasone	Dexamethasone	HD	0.0880
49	01/07/99	10	D	Dexamethasone	Dexamethasone	HD	0.0924
50	01/07/99	11	D	Control	None	HD	0.1052
51	01/07/99	12	D	Control	None	HD	0.1400
52	01/07/99	13	D	Control	None	HD	0.1402

SAP Module II SAS Dataset Created by DBMS Copy from the Microsoft Excel File
KB Module II Summary of SAP.xls - SAP, 010799

Obs	Dose Date	Animal ID	Dose Site	Group	Drug Treatment	Exposure	SAP
1	01/07/99	1	A	Olvanil	Olvanil	HD	0.56847
2	01/07/99	2	A	Olvanil	Olvanil	HD	0.83160
3	01/07/99	3	A	Olvanil	Olvanil	HD	0.35050
4	01/07/99	4	A	Olvanil	Olvanil	HD	0.06538
5	01/07/99	5	A	Olvanil	Olvanil	HD	0.34619
6	01/07/99	6	A	Dexamethasone	Dexamethasone	HD	1.27804
7	01/07/99	7	A	Dexamethasone	Dexamethasone	HD	0.95392
8	01/07/99	8	A	Dexamethasone	Dexamethasone	HD	0.46047
9	01/07/99	9	A	Dexamethasone	Dexamethasone	HD	1.13132
10	01/07/99	10	A	Dexamethasone	Dexamethasone	HD	0.89607
11	01/07/99	11	A	Control	None	HD	0.72683
12	01/07/99	12	A	Control	None	HD	0.21281
13	01/07/99	13	A	Control	None	HD	0.32972
14	01/07/99	14	A	Control	None	HD	0.96297
15	01/07/99	15	A	Control	None	HD	0.87883
16	01/07/99	1	B	Olvanil	None	Sham	0.66326
17	01/07/99	2	B	Olvanil	None	Sham	0.44562
18	01/07/99	3	B	Olvanil	None	Sham	0.23283
19	01/07/99	4	B	Olvanil	None	Sham	0.03653
20	01/07/99	5	B	Olvanil	None	Sham	0.13092
21	01/07/99	6	B	Dexamethasone	None	Sham	0.48853
22	01/07/99	7	B	Dexamethasone	None	Sham	0.15376
23	01/07/99	8	B	Dexamethasone	None	Sham	0.04122
24	01/07/99	9	B	Dexamethasone	None	Sham	0.18168
25	01/07/99	10	B	Dexamethasone	None	Sham	0.22129
26	01/07/99	11	B	Control	None	Sham	0.14792
27	01/07/99	12	B	Control	None	Sham	0.09485
28	01/07/99	13	B	Control	None	Sham	0.16906
29	01/07/99	14	B	Control	None	Sham	0.64953
30	01/07/99	15	B	Control	None	Sham	0.71023
31	01/07/99	1	C	Olvanil	None	Sham	0.68055
32	01/07/99	2	C	Olvanil	None	Sham	0.33492
33	01/07/99	3	C	Olvanil	None	Sham	0.22370
34	01/07/99	4	C	Olvanil	None	Sham	0.03886
35	01/07/99	5	C	Olvanil	None	Sham	0.16149
36	01/07/99	6	C	Dexamethasone	None	Sham	0.64774
37	01/07/99	7	C	Dexamethasone	None	Sham	0.20380
38	01/07/99	8	C	Dexamethasone	None	Sham	0.21389
39	01/07/99	9	C	Dexamethasone	None	Sham	0.20252
40	01/07/99	10	C	Dexamethasone	None	Sham	0.28961
41	01/07/99	11	C	Control	None	Sham	0.27301
42	01/07/99	12	C	Control	None	Sham	0.10962
43	01/07/99	13	C	Control	None	Sham	0.16470
44	01/07/99	14	C	Control	None	Sham	0.63879
45	01/07/99	15	C	Control	None	Sham	0.43548
46	01/07/99	1	D	Olvanil	Olvanil	HD	1.24790
47	01/07/99	2	D	Olvanil	Olvanil	HD	0.83533
48	01/07/99	3	D	Olvanil	Olvanil	HD	0.37819
49	01/07/99	4	D	Olvanil	Olvanil	HD	0.05332
50	01/07/99	5	D	Olvanil	Olvanil	HD	0.37232
51	01/07/99	6	D	Dexamethasone	Dexamethasone	HD	1.23512
52	01/07/99	7	D	Dexamethasone	Dexamethasone	HD	0.76655
53	01/07/99	8	D	Dexamethasone	Dexamethasone	HD	0.69701
54	01/07/99	9	D	Dexamethasone	Dexamethasone	HD	1.19774
55	01/07/99	10	D	Dexamethasone	Dexamethasone	HD	1.48845
56	01/07/99	11	D	Control	None	HD	0.33595
57	01/07/99	12	D	Control	None	HD	0.20396
58	01/07/99	13	D	Control	None	HD	0.32892
59	01/07/99	14	D	Control	None	HD	0.42858
60	01/07/99	15	D	Control	None	HD	.

SAP Module II SAS Dataset Created by DBMS Copy from the Microsoft Excel File
KB Module II Summary of SAP.xls - SAP, 011199

Obs	Dose Date	Animal ID	Dose Site	Group	Drug Treatment	Exposure	SAP
1	01/07/99	1	A	Olvanil	Olvanil	HD	0.35122
2	01/07/99	2	A	Olvanil	Olvanil	HD	1.18255
3	01/07/99	3	A	Olvanil	Olvanil	HD	1.84880
4	01/07/99	4	A	Olvanil	Olvanil	HD	0.37180
5	01/07/99	5	A	Olvanil	Olvanil	HD	1.08133
6	01/07/99	6	A	Dexamethasone	Dexamethasone	HD	1.64228
7	01/07/99	7	A	Dexamethasone	Dexamethasone	HD	0.38897
8	01/07/99	8	A	Dexamethasone	Dexamethasone	HD	0.43433
9	01/07/99	9	A	Dexamethasone	Dexamethasone	HD	0.05633
10	01/07/99	10	A	Dexamethasone	Dexamethasone	HD	0.55173
11	01/07/99	11	A	Control	None	HD	0.50450
12	01/07/99	12	A	Control	None	HD	.
13	01/07/99	13	A	Control	None	HD	0.25666
14	01/07/99	1	B	Olvanil	None	Sham	0.27922
15	01/07/99	2	B	Olvanil	None	Sham	0.05210
16	01/07/99	3	B	Olvanil	None	Sham	1.57529
17	01/07/99	4	B	Olvanil	None	Sham	0.32482
18	01/07/99	5	B	Olvanil	None	Sham	0.05024
19	01/07/99	6	B	Dexamethasone	None	Sham	0.19639
20	01/07/99	7	B	Dexamethasone	None	Sham	0.17340
21	01/07/99	8	B	Dexamethasone	None	Sham	0.20491
22	01/07/99	9	B	Dexamethasone	None	Sham	0.19892
23	01/07/99	10	B	Dexamethasone	None	Sham	0.21766
24	01/07/99	11	B	Control	None	Sham	.
25	01/07/99	12	B	Control	None	Sham	0.08050
26	01/07/99	13	B	Control	None	Sham	0.16811
27	01/07/99	1	C	Olvanil	None	Sham	0.19946
28	01/07/99	2	C	Olvanil	None	Sham	2.08952
29	01/07/99	3	C	Olvanil	None	Sham	1.43163
30	01/07/99	4	C	Olvanil	None	Sham	0.36405
31	01/07/99	5	C	Olvanil	None	Sham	5.33683
32	01/07/99	6	C	Dexamethasone	None	Sham	0.83053
33	01/07/99	7	C	Dexamethasone	None	Sham	0.84221
34	01/07/99	8	C	Dexamethasone	None	Sham	0.70848
35	01/07/99	9	C	Dexamethasone	None	Sham	0.40230
36	01/07/99	10	C	Dexamethasone	None	Sham	0.19274
37	01/07/99	11	C	Control	None	Sham	.
38	01/07/99	12	C	Control	None	Sham	0.05265
39	01/07/99	13	C	Control	None	Sham	0.09906
40	01/07/99	1	D	Olvanil	Olvanil	HD	0.28032
41	01/07/99	2	D	Olvanil	Olvanil	HD	0.08301
42	01/07/99	3	D	Olvanil	Olvanil	HD	1.78971
43	01/07/99	4	D	Olvanil	Olvanil	HD	0.46855
44	01/07/99	5	D	Olvanil	Olvanil	HD	0.13293
45	01/07/99	6	D	Dexamethasone	Dexamethasone	HD	0.27382
46	01/07/99	7	D	Dexamethasone	Dexamethasone	HD	0.33650
47	01/07/99	8	D	Dexamethasone	Dexamethasone	HD	0.20753
48	01/07/99	9	D	Dexamethasone	Dexamethasone	HD	0.52789
49	01/07/99	10	D	Dexamethasone	Dexamethasone	HD	0.61573
50	01/07/99	11	D	Control	None	HD	.
51	01/07/99	12	D	Control	None	HD	0.01125
52	01/07/99	13	D	Control	None	HD	0.56724

Obs	Dose Date	Animal ID	Dose Site	Group	Drug Treatment	Exposure	IL6
1	01/07/99	1	A	Olvanil	Olvanil	HD	0.0910491417
2	01/07/99	2	A	Olvanil	Olvanil	HD	0.358848218
3	01/07/99	3	A	Olvanil	Olvanil	HD	0.298555625
4	01/07/99	4	A	Olvanil	Olvanil	HD	-0.011050915
5	01/07/99	5	A	Olvanil	Olvanil	HD	0.4870531847
6	01/11/99	1	A	Olvanil	Olvanil	HD	0.2447263444
7	01/11/99	2	A	Olvanil	Olvanil	HD	0.3831653502
8	01/11/99	3	A	Olvanil	Olvanil	HD	0.1830671487
9	01/11/99	4	A	Olvanil	Olvanil	HD	0.1772073661
10	01/11/99	5	A	Olvanil	Olvanil	HD	0.2894292181
11	01/07/99	1	D	Olvanil	Olvanil	HD	0.1689683962
12	01/07/99	2	D	Olvanil	Olvanil	HD	0.1054033396
13	01/07/99	3	D	Olvanil	Olvanil	HD	0.1108244273
14	01/07/99	4	D	Olvanil	Olvanil	HD	0.1088041937
15	01/07/99	5	D	Olvanil	Olvanil	HD	0.2825094959
16	01/11/99	1	D	Olvanil	Olvanil	HD	0.4378003054
17	01/11/99	2	D	Olvanil	Olvanil	HD	0.2335313986
18	01/11/99	3	D	Olvanil	Olvanil	HD	0.2710565561
19	01/11/99	4	D	Olvanil	Olvanil	HD	0.2886055091
20	01/11/99	5	D	Olvanil	Olvanil	HD	0.2665587878
21	01/07/99	1	B	Olvanil	None	Sham	-0.031620435
22	01/07/99	2	B	Olvanil	None	Sham	-0.017659726
23	01/07/99	3	B	Olvanil	None	Sham	-0.069995973
24	01/07/99	4	B	Olvanil	None	Sham	-0.069453051
25	01/07/99	5	B	Olvanil	None	Sham	-0.044341062
26	01/11/99	1	B	Olvanil	None	Sham	0.3054615706
27	01/11/99	2	B	Olvanil	None	Sham	0.1963187922
28	01/11/99	3	B	Olvanil	None	Sham	0.2370904445
29	01/11/99	4	B	Olvanil	None	Sham	0.2264682166
30	01/11/99	5	B	Olvanil	None	Sham	0.3134035555
31	01/07/99	1	C	Olvanil	None	Sham	-0.0905462
32	01/07/99	2	C	Olvanil	None	Sham	-0.015950008
33	01/07/99	3	C	Olvanil	None	Sham	-0.054223448
34	01/07/99	4	C	Olvanil	None	Sham	-0.081746879
35	01/07/99	5	C	Olvanil	None	Sham	-0.101488038
36	01/11/99	1	C	Olvanil	None	Sham	0.3668026183
37	01/11/99	2	C	Olvanil	None	Sham	0.267138521
38	01/11/99	3	C	Olvanil	None	Sham	0.2518770772
39	01/11/99	4	C	Olvanil	None	Sham	0.221416395
40	01/11/99	5	C	Olvanil	None	Sham	0.483036054
41	.	.					
42	01/07/99	6	A	Dexamethasone	Dexamethasone	HD	-0.020742391
43	01/07/99	7	A	Dexamethasone	Dexamethasone	HD	-0.011176111
44	01/07/99	8	A	Dexamethasone	Dexamethasone	HD	0.0073914097
45	01/07/99	9	A	Dexamethasone	Dexamethasone	HD	-0.004821255
46	01/07/99	10	A	Dexamethasone	Dexamethasone	HD	-0.007103576
47	01/11/99	6	A	Dexamethasone	Dexamethasone	HD	0.2273400346
48	01/11/99	7	A	Dexamethasone	Dexamethasone	HD	0.1755349849
49	01/11/99	8	A	Dexamethasone	Dexamethasone	HD	0.2018154394
50	01/11/99	9	A	Dexamethasone	Dexamethasone	HD	0.1329084433
51	01/11/99	10	A	Dexamethasone	Dexamethasone	HD	0.0646631716
52	01/07/99	6	D	Dexamethasone	Dexamethasone	HD	-0.120135552
53	01/07/99	7	D	Dexamethasone	Dexamethasone	HD	-0.035920012
54	01/07/99	8	D	Dexamethasone	Dexamethasone	HD	-0.202353552
55	01/07/99	9	D	Dexamethasone	Dexamethasone	HD	-0.036436616
56	01/07/99	10	D	Dexamethasone	Dexamethasone	HD	0.0034715214
57	01/11/99	6	D	Dexamethasone	Dexamethasone	HD	0.407025284
58	01/11/99	7	D	Dexamethasone	Dexamethasone	HD	0.1630613407
59	01/11/99	8	D	Dexamethasone	Dexamethasone	HD	0.2715151467
60	01/11/99	9	D	Dexamethasone	Dexamethasone	HD	0.1636761859
61	01/11/99	10	D	Dexamethasone	Dexamethasone	HD	0.1242824161
62	01/07/99	6	B	Dexamethasone	None	Sham	-0.103779894
63	01/07/99	7	B	Dexamethasone	None	Sham	-0.090394704
64	01/07/99	8	B	Dexamethasone	None	Sham	-0.02125721
65	01/07/99	9	B	Dexamethasone	None	Sham	-0.021802552

IL-6 Module II SAS Dataset Created by DBMS Copy from the Microsoft Excel File
New IL-6 Corrected - IL6 Summary Mod II

Obs	Dose Date	Animal ID	Dose Site	Group	Drug Treatment	Exposure	IL6
66	01/07/99	10	B	Dexamethasone	None	Sham	-0.016430747
67	01/11/99	6	B	Dexamethasone	None	Sham	0.4148696822
68	01/11/99	7	B	Dexamethasone	None	Sham	0.4041763749
69	01/11/99	8	B	Dexamethasone	None	Sham	0.34274951
70	01/11/99	9	B	Dexamethasone	None	Sham	0.2620238023
71	01/11/99	10	B	Dexamethasone	None	Sham	0.1586853364
72	01/07/99	6	C	Dexamethasone	None	Sham	-0.179957798
73	01/07/99	7	C	Dexamethasone	None	Sham	-0.016083881
74	01/07/99	8	C	Dexamethasone	None	Sham	-0.186703708
75	01/07/99	9	C	Dexamethasone	None	Sham	-0.104439522
76	01/07/99	10	C	Dexamethasone	None	Sham	-0.086999693
77	01/11/99	6	C	Dexamethasone	None	Sham	0.2757767429
78	01/11/99	7	C	Dexamethasone	None	Sham	0.3082667723
79	01/11/99	8	C	Dexamethasone	None	Sham	0.1491150681
80	01/11/99	9	C	Dexamethasone	None	Sham	0.134960908
81	01/11/99	10	C	Dexamethasone	None	Sham	0.1482211177
82	.	.					.
83	01/07/99	11	A	Control	None	HD	0.817718658
84	01/07/99	12	A	Control	None	HD	0.3031549477
85	01/07/99	13	A	Control	None	HD	0.4892429156
86	01/07/99	14	A	Control	None	HD	2.6063501608
87	01/07/99	15	A	Control	None	HD	2.5229185329
88	01/11/99	11	A	Control	None	HD	0.2276807907
89	01/11/99	12	A	Control	None	HD	.
90	01/11/99	13	A	Control	None	HD	0.3117653226
91	01/07/99	11	D	Control	None	HD	0.2381330304
92	01/07/99	12	D	Control	None	HD	0.1052074161
93	01/07/99	13	D	Control	None	HD	0.1329122584
94	01/07/99	14	D	Control	None	HD	0.553833021
95	01/07/99	15	D	Control	None	HD	.
96	01/11/99	11	D	Control	None	HD	.
97	01/11/99	12	D	Control	None	HD	0.3365997264
98	01/11/99	13	D	Control	None	HD	0.6017425765
99	01/07/99	11	B	Control	None	Sham	0.0270015547
100	01/07/99	12	B	Control	None	Sham	-0.029265954
101	01/07/99	13	B	Control	None	Sham	-0.058543872
102	01/07/99	14	B	Control	None	Sham	-0.081963501
103	01/07/99	15	B	Control	None	Sham	-0.069650987
104	01/11/99	11	B	Control	None	Sham	.
105	01/11/99	12	B	Control	None	Sham	-0.090676108
106	01/11/99	13	B	Control	None	Sham	-0.046669504
107	01/07/99	11	C	Control	None	Sham	-0.064687345
108	01/07/99	12	C	Control	None	Sham	-0.067054958
109	01/07/99	13	C	Control	None	Sham	-0.044755657
110	01/07/99	14	C	Control	None	Sham	-0.040082432
111	01/07/99	15	C	Control	None	Sham	-0.0627235
112	01/11/99	11	C	Control	None	Sham	.
113	01/11/99	12	C	Control	None	Sham	-0.106291605
114	01/11/99	13	C	Control	None	Sham	-0.028046884

MPX Module II SAS Dataset Created by DBMS Copy from the Microsoft Excel File
MPX ModuleII.xls - 010799

Obs	Dose Date	Animal ID	Dose Site	Group	Drug Treatment	Exposure	Necropsy Time	MPX
1	01/07/99	1	A	Olvanil	Olvanil	HD	24 Hours	0.47511
2	01/07/99	1	B	Olvanil	None	Sham	24 Hours	0.36043
3	01/07/99	1	C	Olvanil	None	Sham	24 Hours	0.40426
4	01/07/99	1	D	Olvanil	Olvanil	HD	24 Hours	0.52300
5	01/07/99	2	A	Olvanil	Olvanil	HD	24 Hours	0.68136
6	01/07/99	2	B	Olvanil	None	Sham	24 Hours	0.51721
7	01/07/99	2	C	Olvanil	None	Sham	24 Hours	0.18714
8	01/07/99	2	D	Olvanil	Olvanil	HD	24 Hours	0.33321
9	01/07/99	3	A	Olvanil	Olvanil	HD	24 Hours	0.64242
10	01/07/99	3	B	Olvanil	None	Sham	24 Hours	0.48616
11	01/07/99	3	C	Olvanil	None	Sham	24 Hours	0.27504
12	01/07/99	3	D	Olvanil	Olvanil	HD	24 Hours	0.31041
13	01/07/99	4	A	Olvanil	Olvanil	HD	24 Hours	0.56558
14	01/07/99	4	B	Olvanil	None	Sham	24 Hours	0.39138
15	01/07/99	4	C	Olvanil	None	Sham	24 Hours	0.29952
16	01/07/99	4	D	Olvanil	Olvanil	HD	24 Hours	0.48263
17	01/07/99	5	A	Olvanil	Olvanil	HD	24 Hours	0.37133
18	01/07/99	5	B	Olvanil	None	Sham	24 Hours	0.78380
19	01/07/99	5	C	Olvanil	None	Sham	24 Hours	0.28568
20	01/07/99	5	D	Olvanil	Olvanil	HD	24 Hours	0.16003
21	01/07/99	6	A	Dexamethasone	Dexamethasone	HD	24 Hours	0.10602
22	01/07/99	6	B	Dexamethasone	None	Sham	24 Hours	0.10717
23	01/07/99	6	C	Dexamethasone	None	Sham	24 Hours	0.05113
24	01/07/99	6	D	Dexamethasone	Dexamethasone	HD	24 Hours	0.06937
25	01/07/99	7	A	Dexamethasone	Dexamethasone	HD	24 Hours	0.20035
26	01/07/99	7	B	Dexamethasone	None	Sham	24 Hours	0.11743
27	01/07/99	7	C	Dexamethasone	None	Sham	24 Hours	0.07157
28	01/07/99	7	D	Dexamethasone	Dexamethasone	HD	24 Hours	0.08554
29	01/07/99	8	A	Dexamethasone	Dexamethasone	HD	24 Hours	0.13091
30	01/07/99	8	B	Dexamethasone	None	Sham	24 Hours	0.08946
31	01/07/99	8	C	Dexamethasone	None	Sham	24 Hours	0.05706
32	01/07/99	8	D	Dexamethasone	Dexamethasone	HD	24 Hours	0.10448
33	01/07/99	9	A	Dexamethasone	Dexamethasone	HD	24 Hours	0.09153
34	01/07/99	9	B	Dexamethasone	None	Sham	24 Hours	0.08249
35	01/07/99	9	C	Dexamethasone	None	Sham	24 Hours	0.10064
36	01/07/99	9	D	Dexamethasone	Dexamethasone	HD	24 Hours	0.08097
37	01/07/99	10	A	Dexamethasone	Dexamethasone	HD	24 Hours	0.30791
38	01/07/99	10	B	Dexamethasone	None	Sham	24 Hours	0.21529
39	01/07/99	10	C	Dexamethasone	None	Sham	24 Hours	0.09023
40	01/07/99	10	D	Dexamethasone	Dexamethasone	HD	24 Hours	0.20975
41	01/07/99	11	A	Control	None	HD	24 Hours	0.86600
42	01/07/99	11	B	Control	None	Sham	24 Hours	0.63195
43	01/07/99	11	C	Control	None	Sham	24 Hours	0.30032
44	01/07/99	11	D	Control	None	HD	24 Hours	0.27235
45	01/07/99	12	A	Control	None	HD	24 Hours	0.76534
46	01/07/99	12	B	Control	None	Sham	24 Hours	0.42631
47	01/07/99	12	C	Control	None	Sham	24 Hours	0.17776
48	01/07/99	12	D	Control	None	HD	24 Hours	0.39514
49	01/07/99	13	A	Control	None	HD	24 Hours	0.83408
50	01/07/99	13	B	Control	None	Sham	24 Hours	0.32281
51	01/07/99	13	C	Control	None	Sham	24 Hours	0.25178
52	01/07/99	13	D	Control	None	HD	24 Hours	0.45347
53	01/07/99	14	A	Control	None	HD	24 Hours	1.33028
54	01/07/99	14	B	Control	None	Sham	24 Hours	1.06861
55	01/07/99	14	C	Control	None	Sham	24 Hours	0.84229
56	01/07/99	14	D	Control	None	HD	24 Hours	1.11912
57	01/07/99	15	A	Control	None	HD	24 Hours	0.77809
58	01/07/99	15	B	Control	None	Sham	24 Hours	0.32656
59	01/07/99	15	C	Control	None	Sham	24 Hours	0.33549
60	01/07/99	15	D	Control	None	HD	24 Hours	0.58678

MPX Module II SAS Dataset Created by DBMS Copy from the Microsoft Excel File
MPX ModuleII.xls - 011199

Obs	Dose Date	Animal ID	Dose Site	Group	Drug Treatment	Exposure	Necropsy Time	MPX
1	01/11/99	1	A	Olvanil	Olvanil	HD	24 Hours	0.52838
2	01/11/99	1	B	Olvanil	None	Sham	24 Hours	0.96491
3	01/11/99	1	C	Olvanil	None	Sham	24 Hours	0.34671
4	01/11/99	1	D	Olvanil	Olvanil	HD	24 Hours	0.40036
5	01/11/99	2	A	Olvanil	Olvanil	HD	24 Hours	0.51943
6	01/11/99	2	B	Olvanil	None	Sham	24 Hours	0.44815
7	01/11/99	2	C	Olvanil	None	Sham	24 Hours	0.19842
8	01/11/99	2	D	Olvanil	Olvanil	HD	24 Hours	0.26119
9	01/11/99	3	A	Olvanil	Olvanil	HD	24 Hours	0.54753
10	01/11/99	3	B	Olvanil	None	Sham	24 Hours	0.29115
11	01/11/99	3	C	Olvanil	None	Sham	24 Hours	0.29192
12	01/11/99	3	D	Olvanil	Olvanil	HD	24 Hours	0.45131
13	01/11/99	4	A	Olvanil	Olvanil	HD	24 Hours	1.23222
14	01/11/99	4	B	Olvanil	None	Sham	24 Hours	0.72990
15	01/11/99	4	C	Olvanil	None	Sham	24 Hours	0.58660
16	01/11/99	4	D	Olvanil	Olvanil	HD	24 Hours	0.54571
17	01/11/99	5	A	Olvanil	Olvanil	HD	24 Hours	0.50879
18	01/11/99	5	B	Olvanil	None	Sham	24 Hours	0.45243
19	01/11/99	5	C	Olvanil	None	Sham	24 Hours	0.15446
20	01/11/99	5	D	Olvanil	Olvanil	HD	24 Hours	0.38523
21	01/11/99	6	A	Dexamethasone	Dexamethasone	HD	24 Hours	0.19763
22	01/11/99	6	B	Dexamethasone	None	Sham	24 Hours	0.10737
23	01/11/99	6	C	Dexamethasone	None	Sham	24 Hours	0.09283
24	01/11/99	6	D	Dexamethasone	Dexamethasone	HD	24 Hours	0.18161
25	01/11/99	7	A	Dexamethasone	Dexamethasone	HD	24 Hours	0.20440
26	01/11/99	7	B	Dexamethasone	None	Sham	24 Hours	0.17518
27	01/11/99	7	C	Dexamethasone	None	Sham	24 Hours	0.19670
28	01/11/99	7	D	Dexamethasone	Dexamethasone	HD	24 Hours	0.22651
29	01/11/99	8	A	Dexamethasone	Dexamethasone	HD	24 Hours	0.48788
30	01/11/99	8	B	Dexamethasone	None	Sham	24 Hours	0.12670
31	01/11/99	8	C	Dexamethasone	None	Sham	24 Hours	0.16307
32	01/11/99	8	D	Dexamethasone	Dexamethasone	HD	24 Hours	0.22408
33	01/11/99	9	A	Dexamethasone	Dexamethasone	HD	24 Hours	0.43043
34	01/11/99	9	B	Dexamethasone	None	Sham	24 Hours	0.25148
35	01/11/99	9	C	Dexamethasone	None	Sham	24 Hours	0.30735
36	01/11/99	9	D	Dexamethasone	Dexamethasone	HD	24 Hours	0.14419
37	01/11/99	10	A	Dexamethasone	Dexamethasone	HD	24 Hours	0.23737
38	01/11/99	10	B	Dexamethasone	None	Sham	24 Hours	0.23021
39	01/11/99	10	C	Dexamethasone	None	Sham	24 Hours	0.21139
40	01/11/99	10	D	Dexamethasone	Dexamethasone	HD	24 Hours	0.24832
41	01/11/99	11	A	Control	None	HD	24 Hours	1.02422
42	01/11/99	11	B	Control	None	Sham	24 Hours	0.66401
43	01/11/99	11	C	Control	None	Sham	24 Hours	0.55050
44	01/11/99	11	D	Control	None	HD	24 Hours	0.80378
45	01/11/99	12	A	Control	None	HD	24 Hours	0.87275
46	01/11/99	12	B	Control	None	Sham	24 Hours	0.51901
47	01/11/99	12	C	Control	None	Sham	24 Hours	0.29671
48	01/11/99	12	D	Control	None	HD	24 Hours	0.71125
49	01/11/99	13	A	Control	None	HD	24 Hours	1.08891
50	01/11/99	13	B	Control	None	Sham	24 Hours	0.31610
51	01/11/99	13	C	Control	None	Sham	24 Hours	0.39811
52	01/11/99	13	D	Control	None	HD	24 Hours	0.92301

APPENDIX I

Module III Statistical Analyses

Battelle
. . . Putting Technology To Work

Project Number G1555-B41ASTAT (3104)

Internal Distribution

Lee/Files
BK Pierce
NA Niemuth
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Date August 3, 2000

To **Carol Sabourin**

From Nancy Niemuth

Subject **Statistical Analysis of Study G1555-41A, MREF Task 95-41 - Module III - Revision 2**

The attached report describes the statistical analysis of tissue weight, SAP, IL-6, and MPX data collected under Module III of Task 95-41. As you noted, the unexposed sites in this module received no treatment (versus sham treatment). Thus, references to sham sites were replaced throughout the report by references to NONE sites. In addition, in preparing supporting documentation for QA review, we noted that the Model 3 results for olvanil and dexamethasone were interchanged in Table 6 for the tissue weight parameter. This table was corrected, as well. An electronic copy of the statistical report will be provided for use in preparing the final report on this study.

Please call me at (614) 424-3231 if you have any questions.

NAN:llj
Attachment

For Review and Approval

	Name	Initials	Date
Originator	Nancy Niemuth	N	8/3/00
Concurrence	Brandon Wood	BJW	8/3/00
Approved	Bill Rosebrough	WRR	8/4/00

Sent: Interoffice mail

Statistical Report for MREF Task 95-41 Module III Data

August 3, 2000

Introduction

Experiments were conducted under Module III of MREF Task 95-41 to determine the efficacy of four drug treatments: ICD 2086 (Indomethacin or IND), ICD 2723 (Olvanil or OLV), ICD 2842 (Hydrocortisone or HC), and ICD 2845 (Dexamethasone or DEX), in moderating the effect of HD exposure at 6 and 24 hours post-exposure, in the mouse ear vesicant model. One exposure site was available on each ear for testing. The right ear was treated (IND, OLV, HC, DEX, or ethanol (ETH) as a control treatment) and exposed to HD. The left ear of each animal was an unexposed (NONE) site. For each exposure time there were 10 animals in each drug treatment group and 15 control animals. There were 5 additional naïve control animals that were not treated or exposed on either ear. The endpoints evaluated were tissue weight, SAP, IL-6, and MPX.

Methods

Data from several animals were not included in the statistical analysis for Module III. The 5 naïve control animals (07/01/98 animals 46-50) were not included in any of the analyses. In addition, many of the control animals were exposed on 8/13/98, approximately 6 weeks after all animals in drug treatment groups and the remaining control animals. All data from 08/13/98 were removed from analyses, as the data from 08/13/98 control animals were found to be statistically significantly different from that of control animals exposed on 06/30/98 and 07/01/98.

A two-stage approach was used to analyze each endpoint, which entailed fitting analysis of variance (ANOVA) models to appropriate subsets of the data. The first model (Model 1) was fitted to data from the unexposed sites. Model 1 tested for a systemic effect due to drug treatment, for each drug treatment compound compared to the control. Based on the results of Model 1 analysis, either Model 2 or Model 3 was selected for the second stage.

If no significant differences were found in Model 1 (indicating there was no systemic effect due to either drug treatment compound) then Model 2 was implemented. Model 2 was fitted to data from the drug treated/HD-exposed sites only. Model 2 tested for differences between HD-exposed sites on drug treated and control animals.

If significant differences were found between unexposed (NONE) site responses for any drug treatment compound as compared to control, indicating a systemic effect due to that treatment compound, Model 3 was employed. Model 3 tested for differences between treatment and control animals after adjusting for differences in unexposed (NONE) site responses. For each animal, the response variable for Model 3 was calculated as the difference between the pretreated/HD-exposed site and the NONE site.

The ANOVA Models 1, 2, and 3 fitted to the tissue weight, SAP, IL-6, and MPX Module III data took the following form:

$$Y_{ij} = \mu + \beta_i + \varepsilon_{ij},$$

where Y_{ij} = tissue weight, SAP, IL-6, or MPX response for j^{th} animal receiving i^{th} treatment
μ = overall average value of the response
β_i = effect of i^{th} treatment
ε = uncontrolled variation.

The ANOVA models were fitted using the SAS (V8) MIXED procedure. For each endpoint, Dunnett's multiple comparison procedure was used to compare each drug treatment group to control. These comparisons are summarized for each model, as follows:

- Model 1: $NONE_{trt} - NONE_{ctl}$
- Model 2: $HD_{trt} - HD_{ctl}$
- Model 3: $(HD_{trt} - NONE_{trt}) - (HD_{ctl} - NONE_{ctl})$

where NONE and HD are the means for NONE sites and pretreated/HD-exposed sites, respectively, for the treatment (trt) or control (ctl) group indicated by the subscript. In addition, the relative response for each group and percent reduction in response for pretreated groups compared to control were calculated as

$$\text{Relative Response} = 100 * (HD - NONE)/ NONE$$

$$\text{Percent Change} = \frac{100 * [(HD_{trt} - NONE_{trt})/ NONE_{trt} - (HD_{ctl} - NONE_{ctl})/ NONE_{ctl}]}{[(HD_{ctl} - NONE_{ctl})/NONE_{ctl}]}$$

Results

Descriptive statistics for tissue weight, SAP, IL-6, and MPX at 6 and 24 hours post-exposure are displayed in Tables 1 through 4, respectively. A summary of the relative response and percent change in response relative to control for all endpoints is provided in Table 5. Parameter estimates and results for the ANOVA models fitted to the tissue weight, SAP, IL-6, and MPX data are presented in Table 7 and summarized below. Model estimated differences between drug treated/HD-exposed sites and NONE sites are presented in Table 7. Listings of the raw data used in the statistical analysis are provided in Appendix A.

A statistically significant increase in tissue weights was associated with HD exposure in the ETH Control group at both 6 and 24 hours (Tables 1 and 7). Model 1 comparisons of NONE sites indicated that there was no systemic effect due to drug treatment at 6 hours post-exposure (Table 6). However, Model 1 did indicate a systemic effect at 24 hours post-exposure for HC and DEX pre-treatments. Thus, Model 3 was fitted to examine the effects of drug treatments on HD-exposed sites for both 6 and 24 hours post-exposure data after adjusting for differences in

NONE site responses. For 6 hours post-exposure data, Model 3 indicated that tissue weight increases for IND, HC, and DEX were statistically significantly less than tissue weight increases in control animals (Table 6). At 24 hours post-exposure, Model 3 indicated that there was a statistically significant reduction in HD-induced response compared to control values for IND. Note that, although tissue weight increases were reduced in drug treated groups compared to controls, tissue weights did increase nearly two-fold compared to NONE sites (Tables 1 and 7).

The effect of HD-exposure on SAP levels in control animals was not statistically significant (Tables 2 and 7). Model 1 comparisons of NONE sites indicated that there was a systemic effect on this parameter due to drug treatment. Significant differences in NONE site responses were noted for HC at 6 hours post-exposure and for all drug treatment compounds at 24 hours post-exposure (Table 6). SAP levels in drug treated animals were approximately 10 ng/mg/mg lower than levels in control animals for NONE sites at 24 hours. Model 3 was used for analysis of 6 and 24 hours post-exposure data. Model 3 indicated no statistically significant differences at 6 hours post-exposure between drug treatments and control SAP values. Model 3 indicated that the IND drug treatment compound resulted in a statistically significant effect on HD-induced response compared to control values at 24 hours post-exposure. As shown in Table 2, the mean SAP levels were similar in HD-exposed and NONE sites for the ethanol group, but increased from 2.7 ng/mg/mg to 38.3 ng/mg/mg in animals treated with IND. A statistically significant increase in SAP values between HD-exposed and NONE sites was found for IND and DEX at 6 hours and for IND at 24 hours post-exposure (Table 7).

IL-6 values at 24 hours post-exposure were consistently less than zero, ranging from –26.6 to –0.04, indicating IL-6 levels were below the limits of detection for the assay. These data were not analyzed. IL-6 levels at 6 hours post-exposure were not increased with exposure to HD in control animals (Tables 3 and 7). Model 1 comparisons of NONE sites indicated a significant systemic effect for DEX and HC drug treatments at 6 hours post-exposure, therefore Model 3 was fitted to the data (Table 6). Model 3 indicated a statistically significant change in HD-induced response compared to control values for OLV treated animals at 6 hours post-exposure. At 6 hours post-exposure, the IL-6 levels were increased in OLV-treated animals (Tables 3 and 7).

The effect of HD-exposure on MPX levels in control animals was not statistically significant (Tables 4 and 7). Model 1 comparisons of NONE sites indicated there was no systemic effect due to drug treatments at 6 hours post-exposure. However, Model 1 indicated that MPX levels were significantly lower at NONE sites for DEX and HC at 24 hours post-exposure. Therefore Model 3 fitted to examine the effects of drug treatment on HD-exposed sites after adjusting for differences in NONE site responses at both 6 and 24 hours post-exposure. Model 3 indicated no statistically significant differences in effect on MPX values at 6 hours post-exposure between drug treatment and ethanol control groups. Model 3 indicated a statistically significant increase in HD-induced response compared to control values for all four drug treatment compounds (IND, OLV, HC, and DEX).

Conclusions

Drug treatments were found to have a systemic effect, indicated by statistically significant differences in NONE site responses, for SAP and IL-6 at 6 hours post-exposure and for all four endpoints (tissue weight, SAP, IL-6, and MPX) at 24 hours post-exposure. These systemic effects were usually due to HC and DEX drug treatments. Drug treatments were effective in moderating the HD-induced response for tissue weight and produced changes in the response for SAP, IL-6 (6 hours), and MPX endpoints. HD-exposure alone caused increases in tissue weights, but did not have a statistically significant effect on SAP, IL-6 (6 hours), or MXP levels.

Table 1. Descriptive Statistics for Tissue Weight for Each Treatment Group at 6 and 24 Hours Post-Exposure

Time Post-Exposure	Group	Ear	Treatment	Exposure	Number of Animals	Tissue Weight (g)			
						Mean	SD	Relative Response[1]	Percent Change[2]
6 hr	Control	L	None	NONE	5	0.014	0.002	57	NA
		R	ETH	HD		0.023	0.003		
	Indomethacin	L	None	NONE	10	0.015	0.001	24	-57
		R	IND	HD		0.019	0.003		
	Olvanil	L	None	NONE	10	0.015	0.001	51	-10
		R	OLV	HD		0.023	0.004		
	Hydrocortisone	L	None	NONE	10	0.014	0.001	13	-76
		R	HC	HD		0.016	0.002		
	Dexamethasone	L	None	NONE	10	0.015	0.001	12	-78
		R	DEX	HD		0.017	0.001		
24 hr	Control	L	None	NONE	5	0.016	0.001	158	NA
		R	ETH	HD		0.041	0.003		
	Indomethacin	L	None	NONE	10	0.016	0.001	101	-36
		R	IND	HD		0.031	0.005		
	Olvanil	L	None	NONE	10	0.015	0.001	132	-17
		R	OLV	HD		0.034	0.008		
	Hydrocortisone	L	None	NONE	10	0.014	0.001	136	-14
		R	HC	HD		0.034	0.006		
	Dexamethasone	L	None	NONE	10	0.013	0.001	144	-9
		R	DEX	HD		0.032	0.006		

[1] **Relative Response = 100 * (HD – NONE)/ NONE.**

[2] **Percent Change = 100 * $[(HD_{trt} - NONE_{trt})/ NONE_{trt} - (HD_{ctl} - NONE_{ctl})/ NONE_{ctl}]/[(HD_{ctl} - NONE_{ctl})/ NONE_{ctl}]$.**

Table 2. Descriptive Statistics for SAP for Each Treatment Group at 6 and 24 Hours Post-Exposure

Time Post-Exposure	Group	Ear	Treatment	Exposure	Number of Animals	SAP (ng/mg/mg)			
						Mean	SD	Relative Response[1]	Percent Change[2]
6 hr	Control	L	None	NONE	5	0.432	0.142	202	NA
		R	ETH	HD		1.304	0.732		
	Indomethacin	L	None	NONE	10	1.262	0.825	82	-60
		R	IND	HD		2.295	1.447		
	Olvanil	L	None	NONE	10	1.176	1.484	67	-67
		R	OLV	HD		1.959	1.547		
	Hydrocortisone	L	None	NONE	10	2.877	1.131	16	-92
		R	HC	HD		3.343	2.574		
	Dexamethasone	L	None	NONE	10	2.021	1.548	65	-68
		R	DEX	HD		3.332	2.060		
24 hr	Control	L	None	NONE	5	13.228	22.306	10	NA
		R	ETH	HD		14.489	6.688		
	Indomethacin	L	None	NONE	10	2.737	1.321	1301	13535
		R	IND	HD		38.341	22.270		
	Olvanil	L	None	NONE	10	2.049	1.138	264	2671
		R	OLV	HD		7.463	9.776		
	Hydrocortisone	L	None	NONE	10	2.820	3.852	89	838
		R	HC	HD		5.344	3.230		
	Dexamethasone	L	None	NONE	10	1.813	0.991	234	2352
		R	DEX	HD		6.053	6.104		

[1] Relative Response = 100 * (HD – NONE)/ NONE.

[2] Percent Change = 100 * $[(HD_{trt} - NONE_{trt})/ NONE_{trt} - (HD_{ctl} - NONE_{ctl})/ NONE_{ctl}]/[(HD_{ctl} - NONE_{ctl})/ NONE_{ctl}]$.

Table 3. Descriptive Statistics for IL-6 for Each Treatment Group at 6 and 24 Hours Post-Exposure

Time Post-Exposure	Group	Ear	Treatment	Exposure	Number of Animals	IL-6 (pg/mg/mg)			
						Mean	SD	Relative Response[1]	Percent Change[2]
6 hr	Control	L	None	NONE	5	2.081	2.172	-43	NA
		R	ETH	HD		1.180	0.661		
	Indomethacin	L	None	NONE	9	1.622	0.701	-62	-242
		R	IND	HD		2.620	1.193		
	Olvanil	L	None	NONE	10	2.476	1.392	212	-591
		R	OLV	HD		7.735	2.535		
	Hydrocortisone	L	None	NONE	10	8.245	3.201	16	-138
		R	HC	HD		9.603	4.523		
	Dexamethasone	L	None	NONE	10	9.644	2.008	-18	-58
		R	DEX	HD		7.883	2.717		
24 hr	Control	L	None	NONE	5	3.069	3.290	-76	NA
		R	ETH	HD		0.725	0.368		
	Indomethacin	L	None	NONE	10	-5.483	3.785	-116	52
		R	IND	HD		0.887	1.301		
	Olvanil	L	None	NONE	10	-8.467	4.179	-95	24
		R	OLV	HD		-0.417	1.002		
	Hydrocortisone	L	None	NONE	10	-3.365	0.889	-121	58
		R	HC	HD		0.700	0.620		
	Dexamethasone	L	None	NONE	10	-7.352	7.244	-94	23
		R	DEX	HD		-0.435	0.732		

[1] **Relative Response = 100 * (HD – NONE)/ NONE.**

[2] **Percent Change = 100 * $[(HD_{trt} - NONE_{trt})/ NONE_{trt} - (HD_{ctl} - NONE_{ctl})/ NONE_{ctl}]/[(HD_{ctl} - NONE_{ctl})/ NONE_{ctl}]$.**

Table 4. Descriptive Statistics for MPX for Each Treatment Group at 6 and 24 Hours Post-Exposure

Time Post-Exposure	Group	Ear	Treatment	Exposure	Number of Animals	MPX (U/mg)			
						Mean	SD	Relative Response[1]	Percent Change[2]
6 hr	Control	L	None	NONE	5	0.459	0.286	68	NA
		R	ETH	HD		0.769	0.136		
	Indomethacin	L	None	NONE	9	0.631	0.533	154	127
		R	IND	HD		1.599	0.717		
	Olvanil	L	None	NONE	10	0.830	0.573	87	28
		R	OLV	HD		1.549	0.513		
	Hydrocortisone	L	None	NONE	10	0.417	0.269	200	196
		R	HC	HD		1.251	0.659		
	Dexamethasone	L	None	NONE	10	0.597	0.411	56	-16
		R	DEX	HD		0.937	0.306		
24 hr	Control	L	None	NONE	5	2.251	4.200	-48	NA
		R	ETH	HD		1.160	0.313		
	Indomethacin	L	None	NONE	10	0.496	0.204	264	-646
		R	IND	HD		1.810	0.942		
	Olvanil	L	None	NONE	10	0.578	0.471	223	-560
		R	OLV	HD		1.865	0.688		
	Hydrocortisone	L	None	NONE	10	0.362	0.147	376	-876
		R	HC	HD		1.724	0.648		
	Dexamethasone	L	None	NONE	10	0.169	0.122	625	-1389
		R	DEX	HD		1.227	0.472		

[1] **Relative Response = 100 * (HD – NONE)/ NONE.**

[2] **Percent Change = 100 * $[(HD_{trt} - NONE_{trt})/ NONE_{trt} - (HD_{ctl} - NONE_{ctl})/ NONE_{ctl}]/[(HD_{ctl} - NONE_{ctl})/ NONE_{ctl}]$.**

Table 5. Relative Response and Percent Reduction in Response Relative to Control for Tissue Weight, SAP, IL-6, and MPX Data Collected in Module III

Endpoint	Time Post-Exposure	Group	Relative Response[1] (%)	Percent Change Relative to Control[2] (%)
Tissue Weight	6 hr	Control	57	0
		Indomethacin	24	-57
		Olvanil	51	-10
		Hydrocortisone	13	-76
		Dexamethasone	12	-78
	24 hr	Control	158	0
		Indomethacin	101	-36
		Olvanil	132	-17
		Hydrocortisone	136	-14
		Dexamethasone	144	-9
SAP	6 hr	Control	202	0
		Indomethacin	82	-60
		Olvanil	67	-67
		Hydrocortisone	16	-92
		Dexamethasone	65	-68
	24 hr	Control	10	0
		Indomethacin	1301	13535
		Olvanil	264	2671
		Hydrocortisone	89	838
		Dexamethasone	234	2352
IL-6	6 hr	Control	-43	0
		Indomethacin	62	-242
		Olvanil	212	-591
		Hydrocortisone	16	-138
		Dexamethasone	-18	-58
	24 hr	Control	-76	0
		Indomethacin	-116	52
		Olvanil	-95	24
		Hydrocortisone	-121	58
		Dexamethasone	-94	23
MPX	6 hr	Control	68	0
		Indomethacin	154	127
		Olvanil	87	28
		Hydrocortisone	200	196
		Dexamethasone	57	-16
	24 hr	Control	-48	0
		Indomethacin	265	-646
		Olvanil	223	-560
		Hydrocortisone	376	-876
		Dexamethasone	625	-1389

[1] Relative Response = 100 * (HD – NONE)/ NONE.

[2] Percent Change = 100 * $[(HD_{trt} - NONE_{trt})/ NONE_{trt} - (HD_{ctl} - NONE_{ctl})/ NONE_{ctl}]/[(HD_{ctl} - NONE_{ctl})/ NONE_{ctl}]$.

Table 6. Model Estimated Differences between Treatment Groups for Tissue Weight, SAP, IL-6, and MPX, by Exposure Time

Endpoint	Time Post-Exposure	Comparison	Model 1 Results			Model Chosen	Final Model Results		
			Estimated Difference (NONE sites)	SE	p-Value		Estimated Difference Due to Treatment	SE	p-Value
Tissue Weight	6 hr	IND vs Control	0.001	0.001	0.245	3	-0.004	0.001	0.006
		OLV vs Control	0.001	0.001	0.390		-0.0004	0.001	0.989
		HC vs Control	-0.0001	0.001	1.000		-0.006	0.001	<0.001
		DEX vs Control	0.001	0.001	0.438		-0.005	0.001	<0.001
	24 hr	IND vs Control	-0.001	0.001	0.762	3	-0.010	0.003	0.011
		OLV vs Control	-0.001	0.001	0.124		-0.006	0.003	0.175
		HC vs Control	-0.002	0.001	0.027		-0.006	0.003	0.180
		DEX vs Control	-0.003	0.001	<0.001		-0.006	0.003	0.127
SAP	6 hr	IND vs Control	0.830	0.683	0.504	3	0.160	0.756	0.998
		OLV vs Control	0.744	0.671	0.576		-0.090	0.742	1.000
		HC vs Control	2.446	0.671	0.003		-0.407	0.742	0.929
		DEX vs Control	1.589	0.671	0.065		0.438	0.742	0.911
	24 hr	IND vs Control	-10.490	4.128	0.045	3	34.342	7.076	<0.001
		OLV vs Control	-11.179	4.128	0.031		4.153	7.076	0.914
		HC vs Control	-10.408	4.128	0.047		1.262	7.076	0.999
		DEX vs Control	-11.415	4.296	0.035		2.979	7.365	0.975
IL-6[1]	6 hr	IND vs Control	-0.459	1.160	0.976	3	1.898	1.473	0.457
		OLV vs Control	0.395	1.139	0.985		6.160	1.447	<0.001
		HC vs Control	6.164	1.139	<0.001		2.259	1.447	0.304
		DEX vs Control	7.564	1.139	<0.001		-0.861	1.447	0.908
MPX	6 hr	IND vs Control	0.172	0.248	0.858	3	0.658	0.353	0.180
		OLV vs Control	0.371	0.244	0.324		0.409	0.346	0.526
		HC vs Control	-0.042	0.244	0.999		0.524	0.346	0.328
		DEX vs Control	0.139	0.244	0.920		0.029	0.346	1.000
	24 hr	IND vs Control	-1.755	0.741	0.065	3	2.406	0.807	0.015
		OLV vs Control	-1.674	0.741	0.082		2.380	0.807	0.017
		HC vs Control	-1.889	0.741	0.043		2.454	0.807	0.013
		DEX vs Control	-2.082	0.741	0.023		2.149	0.807	0.033

[1] **As IL-6 values at 24 hours post-exposure were consistently less than zero, indicating levels below the limits of detection for the assay, these data were not analyzed.**

Table 7. Model Estimated Differences between HD-Exposed Sites and NONE Sites within Each Treatment Group

Endpoint	Time Post-Exposure	Group	Estimated Difference (HD-NONE)	SE	p-Value (T-Test)
Tissue Weight	6 hr	Control	0.008	0.001	<0.001
		IND	0.004	0.001	<0.001
		OLV	0.008	0.001	<0.001
		HC	0.002	0.001	0.016
		DEX	0.002	0.001	0.017
	24 hr	Control	0.025	0.003	<0.001
		IND	0.016	0.002	<0.001
		OLV	0.020	0.002	<0.001
		HC	0.020	0.002	<0.001
		DEX	0.019	0.002	<0.001
SAP	6 hr	Control	0.873	0.606	0.158
		IND	1.033	0.452	0.028
		OLV	0.783	0.428	0.075
		HC	0.466	0.428	0.283
		DEX	1.311	0.428	0.004
	24 hr	Control	1.262	5.777	0.828
		IND	35.603	4.085	<0.001
		OLV	5.414	4.085	0.193
		HC	2.524	4.085	0.540
		DEX	4.240	4.567	0.359
IL-6[1]	6 hr	Control	-0.900	1.181	0.451
		IND	0.998	0.881	0.264
		OLV	5.259	0.835	<0.001
		HC	1.359	0.835	0.112
		DEX	-1.761	0.835	0.041
MPX	6 hr	Control	0.311	0.283	0.279
		IND	0.969	0.211	<0.001
		OLV	0.720	0.200	<0.001
		HC	0.834	0.200	<0.001
		DEX	0.339	0.200	0.098
	24 hr	Control	-1.092	0.659	0.105
		IND	1.314	0.466	0.007
		OLV	1.288	0.466	0.009
		HC	1.362	0.466	0.006
		DEX	1.058	0.466	0.029

[1] **As IL-6 values at 24 hours post-exposure were consistently less than zero, indicating levels below the limits of detection for the assay, these data were not analyzed.**

APPENDIX A:

LISTINGS OF ANALYSIS DATASETS

Tissue Weight from SAP Module III SAS Dataset Created by DBMS Copy from the Microsoft Excel File SAP Data.1.xls - SAP

Obs	DoseDate	Animal ID	Dose Site	Group	PreTreatment	Exposure	Necropsy Time	Tissue Weight
1	08/13/98	1	Right	Control	Ethanol	HD	6	0.0152
2	08/13/98	1	Left	Control	None	NONE	6	0.0127
3	08/13/98	2	Right	Control	Ethanol	HD	6	0.0173
4	08/13/98	2	Left	Control	None	NONE	6	0.0134
5	08/13/98	3	Right	Control	Ethanol	HD	6	0.0152
6	08/13/98	3	Left	Control	None	NONE	6	0.0125
7	08/13/98	4	Right	Control	Ethanol	HD	6	0.0153
8	08/13/98	4	Left	Control	None	NONE	6	0.0121
9	08/13/98	5	Right	Control	Ethanol	HD	6	0.0157
10	08/13/98	5	Left	Control	None	NONE	6	0.0118
11	08/13/98	6	Right	Control	Ethanol	HD	6	0.0178
12	08/13/98	6	Left	Control	None	NONE	6	0.0126
13	08/13/98	7	Right	Control	Ethanol	HD	6	0.0145
14	08/13/98	7	Left	Control	None	NONE	6	0.0135
15	08/13/98	8	Right	Control	Ethanol	HD	6	0.0157
16	08/13/98	8	Left	Control	None	NONE	6	0.0127
17	08/13/98	9	Right	Control	Ethanol	HD	6	0.0191
18	08/13/98	9	Left	Control	None	NONE	6	0.0143
19	08/13/98	10	Right	Control	Ethanol	HD	6	0.0152
20	08/13/98	10	Left	Control	None	NONE	6	0.0120
21	08/13/98	29	Right	Control	Ethanol	HD	24	0.0420
22	08/13/98	29	Left	Control	None	NONE	24	0.0147
23	08/13/98	30	Right	Control	Ethanol	HD	24	0.0426
24	08/13/98	30	Left	Control	None	NONE	24	0.0131
25	08/13/98	31	Right	Control	Ethanol	HD	24	0.0382
26	08/13/98	31	Left	Control	None	NONE	24	0.0137
27	08/13/98	32	Right	Control	Ethanol	HD	24	0.0344
28	08/13/98	32	Left	Control	None	NONE	24	0.0127
29	08/13/98	33	Right	Control	Ethanol	HD	24	0.0411
30	08/13/98	33	Left	Control	None	NONE	24	0.0132
31	08/13/98	34	Right	Control	Ethanol	HD	24	0.0443
32	08/13/98	34	Left	Control	None	NONE	24	0.0155
33	08/13/98	35	Right	Control	Ethanol	HD	24	0.0416
34	08/13/98	35	Left	Control	None	NONE	24	0.0143
35	08/13/98	36	Right	Control	Ethanol	HD	24	0.0415
36	08/13/98	36	Left	Control	None	NONE	24	0.0144
37	08/13/98	37	Right	Control	Etahnol	HD	24	0.0329
38	08/13/98	37	Left	Control	None	NONE	24	0.0132
39	08/13/98	38	Right	Control	Ethanol	HD	24	0.0372
40	08/13/98	38	Left	Control	None	NONE	24	0.0129
41	07/01/98	1	Right	Hydrocortisone	Hydrocortisone	HD	24	0.0335
42	07/01/98	1	Left	Hydrocortisone	None	NONE	24	0.0157
43	07/01/98	2	Right	Hydrocortisone	Hydrocortisone	HD	24	0.0279
44	07/01/98	2	Left	Hydrocortisone	None	NONE	24	0.0142
45	07/01/98	3	Right	Hydrocortisone	Hydrocortisone	HD	24	0.0381
46	07/01/98	3	Left	Hydrocortisone	None	NONE	24	0.0133
47	07/01/98	4	Right	Hydrocortisone	Hydrocortisone	HD	24	0.0332
48	07/01/98	4	Left	Hydrocortisone	None	NONE	24	0.0139
49	07/01/98	5	Right	Hydrocortisone	Hydrocortisone	HD	24	0.0229
50	07/01/98	5	Left	Hydrocortisone	None	NONE	24	0.0140
51	07/01/98	6	Right	Hydrocortisone	Hydrocortisone	HD	24	0.0366
52	07/01/98	6	Left	Hydrocortisone	None	NONE	24	0.0149
53	07/01/98	7	Right	Hydrocortisone	Hydrocortisone	HD	24	0.0343
54	07/01/98	7	Left	Hydrocortisone	None	NONE	24	0.0147
55	07/01/98	8	Right	Hydrocortisone	Hydrocortisone	HD	24	0.0322
56	07/01/98	8	Left	Hydrocortisone	None	NONE	24	0.0137
57	07/01/98	9	Right	Hydrocortisone	Hydrocortisone	HD	24	0.0429
58	07/01/98	9	Left	Hydrocortisone	None	NONE	24	0.0148
59	07/01/98	10	Right	Hydrocortisone	Hydrocortisone	HD	24	0.0379
60	07/01/98	10	Left	Hydrocortisone	None	NONE	24	0.0148
61	07/01/98	11	Right	Dexamethasone	Dexamethasone	HD	24	0.0203
62	07/01/98	11	Left	Dexamethasone	None	NONE	24	0.0124
63	07/01/98	12	Right	Dexamethasone	Dexamethasone	HD	24	0.0295
64	07/01/98	12	Left	Dexamethasone	None	NONE	24	0.0118
65	07/01/98	13	Right	Dexamethasone	Dexamethasone	HD	24	0.0314
66	07/01/98	13	Left	Dexamethasone	None	NONE	24	0.0128
67	07/01/98	14	Right	Dexamethasone	Dexamethasone	HD	24	0.0376
68	07/01/98	14	Left	Dexamethasone	None	NONE	24	0.0133
69	07/01/98	15	Right	Dexamethasone	Dexamethasone	HD	24	0.0258
70	07/01/98	15	Left	Dexamethasone	None	NONE	24	0.0120
71	07/01/98	16	Right	Dexamethasone	Dexamethasone	HD	24	0.0339

Tissue Weight from SAP Module III SAS Dataset Created by DBMS Copy from the Microsoft Excel File SAP Data.1.xls - SAP

Obs	DoseDate	Animal ID	Dose Site	Group	PreTreatment	Exposure	Necropsy Time	Tissue Weight
72	07/01/98	16	Left	Dexamethasone	None	NONE	24	0.0137
73	07/01/98	17	Right	Dexamethasone	Dexamethasone	HD	24	0.0403
74	07/01/98	17	Left	Dexamethasone	None	NONE	24	0.0139
75	07/01/98	18	Right	Dexamethasone	Dexamethasone	HD	24	0.0337
76	07/01/98	18	Left	Dexamethasone	None	NONE	24	0.0152
77	07/01/98	19	Right	Dexamethasone	Dexamethasone	HD	24	0.0346
78	07/01/98	19	Left	Dexamethasone	None	NONE	24	0.0140
79	07/01/98	20	Right	Dexamethasone	Dexamethasone	HD	24	0.0346
80	07/01/98	20	Left	Dexamethasone	None	NONE	24	0.0128
81	07/01/98	21	Right	Olvanil	Olvanil	HD	24	0.0219
82	07/01/98	21	Left	Olvanil	None	NONE	24	0.0145
83	07/01/98	22	Right	Olvanil	Olvanil	HD	24	0.0241
84	07/01/98	22	Left	Olvanil	None	NONE	24	0.0140
85	07/01/98	23	Right	Olvanil	Olvanil	HD	24	0.0460
86	07/01/98	23	Left	Olvanil	None	NONE	24	0.0164
87	07/01/98	24	Right	Olvanil	Olvanil	HD	24	0.0446
88	07/01/98	24	Left	Olvanil	None	NONE	24	0.0148
89	07/01/98	25	Right	Olvanil	Olvanil	HD	24	0.0278
90	07/01/98	25	Left	Olvanil	None	NONE	24	0.0158
91	07/01/98	26	Right	Olvanil	Olvanil	HD	24	0.0360
92	07/01/98	26	Left	Olvanil	None	NONE	24	0.0144
93	07/01/98	27	Right	Olvanil	Olvanil	HD	24	0.0385
94	07/01/98	27	Left	Olvanil	None	NONE	24	0.0164
95	07/01/98	28	Right	Olvanil	Olvanil	HD	24	0.0328
96	07/01/98	28	Left	Olvanil	None	NONE	24	0.0145
97	07/01/98	29	Right	Olvanil	Olvanil	HD	24	0.0333
98	07/01/98	29	Left	Olvanil	None	NONE	24	0.0129
99	07/01/98	30	Right	Olvanil	Olvanil	HD	24	0.0381
100	07/01/98	30	Left	Olvanil	None	NONE	24	0.0143
101	07/01/98	31	Right	Indomethacin	Indomethacin	HD	24	0.0310
102	07/01/98	31	Left	Indomethacin	None	NONE	24	0.0136
103	07/01/98	32	Right	Indomethacin	Indomethacin	HD	24	0.0333
104	07/01/98	32	Left	Indomethacin	None	NONE	24	0.0169
105	07/01/98	33	Right	Indomethacin	Indomethacin	HD	24	0.0316
106	07/01/98	33	Left	Indomethacin	None	NONE	24	0.0176
107	07/01/98	34	Right	Indomethacin	Indomethacin	HD	24	0.0289
108	07/01/98	34	Left	Indomethacin	None	NONE	24	0.0160
109	07/01/98	35	Right	Indomethacin	Indomethacin	HD	24	0.0316
110	07/01/98	35	Left	Indomethacin	None	NONE	24	0.0157
111	07/01/98	36	Right	Indomethacin	Indomethacin	HD	24	0.0441
112	07/01/98	36	Left	Indomethacin	None	NONE	24	0.0161
113	07/01/98	37	Right	Indomethacin	Indomethacin	HD	24	0.0264
114	07/01/98	37	Left	Indomethacin	None	NONE	24	0.0156
115	07/01/98	38	Right	Indomethacin	Indomethacin	HD	24	0.0299
116	07/01/98	38	Left	Indomethacin	None	NONE	24	0.0130
117	07/01/98	39	Right	Indomethacin	Indomethacin	HD	24	0.0274
118	07/01/98	39	Left	Indomethacin	None	NONE	24	0.0151
119	07/01/98	40	Right	Indomethacin	Indomethacin	HD	24	0.0274
120	07/01/98	40	Left	Indomethacin	None	NONE	24	0.0155
121	07/01/98	41	Right	Control	Ethanol	HD	24	0.0393
122	07/01/98	41	Left	Control	None	NONE	24	0.0146
123	07/01/98	42	Right	Control	Ethanol	HD	24	0.0419
124	07/01/98	42	Left	Control	None	NONE	24	0.0160
125	07/01/98	43	Right	Control	Ethanol	HD	24	0.0385
126	07/01/98	43	Left	Control	None	NONE	24	0.0159
127	07/01/98	44	Right	Control	Ethanol	HD	24	0.0448
128	07/01/98	44	Left	Control	None	NONE	24	0.0168
129	07/01/98	45	Right	Control	Ethanol	HD	24	0.0421
130	07/01/98	45	Left	Control	None	NONE	24	0.0167
131	07/01/98	46	Right	Naive	None	NONE	24	0.0146
132	07/01/98	46	Left	Naive	None	NONE	24	0.0147
133	07/01/98	47	Right	Naive	None	NONE	24	0.0160
134	07/01/98	47	Left	Naive	None	NONE	24	0.0159
135	07/01/98	48	Right	Naive	None	NONE	24	0.0160
136	07/01/98	48	Left	Naive	None	NONE	24	0.0173
137	07/01/98	49	Right	Naive	None	NONE	24	0.0139
138	07/01/98	49	Left	Naive	None	NONE	24	0.0141
139	07/01/98	50	Right	Naive	None	NONE	24	0.0151
140	07/01/98	50	Left	Naive	None	NONE	24	0.0155
141	06/30/98	1	Right	Hydrocortisone	Hydrocortisone	HD	6	0.0171
142	06/30/98	1	Left	Hydrocortisone	None	NONE	6	0.0144

Tissue Weight from SAP Module III SAS Dataset Created by DBMS Copy from the Microsoft Excel File SAP Data.1.xls - SAP

Obs	DoseDate	Animal ID	Dose Site	Group	PreTreatment	Exposure	Necropsy Time	Tissue Weight
143	06/30/98	2	Right	Hydrocortisone	Hydrocortisone	HD	6	0.0184
144	06/30/98	2	Left	Hydrocortisone	None	NONE	6	0.0155
145	06/30/98	3	Right	Hydrocortisone	Hydrocortisone	HD	6	0.0151
146	06/30/98	3	Left	Hydrocortisone	None	NONE	6	0.0140
147	06/30/98	4	Right	Hydrocortisone	Hydrocortisone	HD	6	0.0137
148	06/30/98	4	Left	Hydrocortisone	None	NONE	6	0.0119
149	06/30/98	5	Right	Hydrocortisone	Hydrocortisone	HD	6	0.0149
150	06/30/98	5	Left	Hydrocortisone	None	NONE	6	0.0126
151	06/30/98	6	Right	Hydrocortisone	Hydrocortisone	HD	6	0.0186
152	06/30/98	6	Left	Hydrocortisone	None	NONE	6	0.0155
153	06/30/98	7	Right	Hydrocortisone	Hydrocortisone	HD	6	0.0156
154	06/30/98	7	Left	Hydrocortisone	None	NONE	6	0.0151
155	06/30/98	8	Right	Hydrocortisone	Hydrocortisone	HD	6	0.0165
156	06/30/98	8	Left	Hydrocortisone	None	NONE	6	0.0146
157	06/30/98	9	Right	Hydrocortisone	Hydrocortisone	HD	6	0.0156
158	06/30/98	9	Left	Hydrocortisone	None	NONE	6	0.0145
159	06/30/98	10	Right	Hydrocortisone	Hydrocortisone	HD	6	0.0172
160	06/30/98	10	Left	Hydrocortisone	None	NONE	6	0.0155
161	06/30/98	11	Right	Dexamethasone	Dexamethasone	HD	6	0.0158
162	06/30/98	11	Left	Dexamethasone	None	NONE	6	0.0143
163	06/30/98	12	Right	Dexamethasone	Dexamethasone	HD	6	0.0158
164	06/30/98	12	Left	Dexamethasone	None	NONE	6	0.0144
165	06/30/98	13	Right	Dexamethasone	Dexamethasone	HD	6	0.0171
166	06/30/98	13	Left	Dexamethasone	None	NONE	6	0.0159
167	06/30/98	14	Right	Dexamethasone	Dexamethasone	HD	6	0.0185
168	06/30/98	14	Left	Dexamethasone	None	NONE	6	0.0167
169	06/30/98	15	Right	Dexamethasone	Dexamethasone	HD	6	0.0166
170	06/30/98	15	Left	Dexamethasone	None	NONE	6	0.0147
171	06/30/98	16	Right	Dexamethasone	Dexamethasone	HD	6	0.0177
172	06/30/98	16	Left	Dexamethasone	None	NONE	6	0.0155
173	06/30/98	17	Right	Dexamethasone	Dexamethasone	HD	6	0.0170
174	06/30/98	17	Left	Dexamethasone	None	NONE	6	0.0154
175	06/30/98	18	Right	Dexamethasone	Dexamethasone	HD	6	0.0182
176	06/30/98	18	Left	Dexamethasone	None	NONE	6	0.0158
177	06/30/98	19	Right	Dexamethasone	Dexamethasone	HD	6	0.0164
178	06/30/98	19	Left	Dexamethasone	None	NONE	6	0.0142
179	06/30/98	20	Right	Dexamethasone	Dexamethasone	HD	6	0.0183
180	06/30/98	20	Left	Dexamethasone	None	NONE	6	0.0156
181	06/30/98	21	Right	Olvanil	Olvanil	HD	6	0.0227
182	06/30/98	21	Left	Olvanil	None	NONE	6	0.0151
183	06/30/98	22	Right	Olvanil	Olvanil	HD	6	0.0252
184	06/30/98	22	Left	Olvanil	None	NONE	6	0.0167
185	06/30/98	23	Right	Olvanil	Olvanil	HD	6	0.0179
186	06/30/98	23	Left	Olvanil	None	NONE	6	0.0135
187	06/30/98	24	Right	Olvanil	Olvanil	HD	6	0.0274
188	06/30/98	24	Left	Olvanil	None	NONE	6	0.0157
189	06/30/98	25	Right	Olvanil	Olvanil	HD	6	0.0274
190	06/30/98	25	Left	Olvanil	None	NONE	6	0.0169
191	06/30/98	26	Right	Olvanil	Olvanil	HD	6	0.0177
192	06/30/98	26	Left	Olvanil	None	NONE	6	0.0163
193	06/30/98	27	Right	Olvanil	Olvanil	HD	6	0.0247
194	06/30/98	27	Left	Olvanil	None	NONE	6	0.0138
195	06/30/98	28	Right	Olvanil	Olvanil	H D	6	0.0256
196	06/30/98	28	Left	Olvanil	None	NONE	6	0.0159
197	06/30/98	29	Right	Olvanil	Olvanil	HD	6	0.0173
198	06/30/98	29	Left	Olvanil	None	NONE	6	0.0140
199	06/30/98	30	Right	Olvanil	Olvanil	HD	6	0.0245
200	06/30/98	30	Left	Olvanil	None	NONE	6	0.0151
201	06/30/98	31	Right	Indomethacin	Indomethacin	HD	6	0.0167
202	06/30/98	31	Left	Indomethacin	None	NONE	6	0.0152
203	06/30/98	32	Right	Indomethacin	Indomethacin	HD	6	0.0227
204	06/30/98	32	Left	Indomethacin	None	NONE	6	0.0147
205	06/30/98	33	Right	Indomethacin	Indomethacin	HD	6	0.0233
206	06/30/98	33	Left	Indomethacin	None	NONE	6	0.0158
207	06/30/98	34	Right	Indomethacin	Indomethacin	HD	6	0.0175
208	06/30/98	34	Left	Indomethacin	None	NONE	6	0.0166
209	06/30/98	35	Right	Indomethacin	Indomethacin	HD	6	0.0207
210	06/30/98	35	Left	Indomethacin	None	NONE	6	0.0166
211	06/30/98	37	Right	Indomethacin	Indomethacin	HD	6	0.0226
212	06/30/98	37	Left	Indomethacin	None	NONE	6	0.0144
213	06/30/98	38	Right	Indomethacin	Indomethacin	HD	6	0.0180

Tissue Weight from SAP Module III SAS Dataset Created by DBMS Copy from the Microsoft Excel File SAP Data.1.xls - SAP

Obs	DoseDate	Animal ID	Dose Site	Group	PreTreatment	Exposure	Necropsy Time	Tissue Weight
214	06/30/98	38	Left	Indomethacin	None	NONE	6	0.0151
215	06/30/98	39	Right	Indomethacin	Indomethacin	HD	6	0.0159
216	06/30/98	39	Left	Indomethacin	None	NONE	6	0.0145
217	06/30/98	40	Right	Indomethacin	Indomethacin	HD	6	0.0162
218	06/30/98	40	Left	Indomethacin	None	NONE	6	0.0155
219	06/30/98	41	Right	Control	Ethanol	HD	6	0.0216
220	06/30/98	41	Left	Control	None	NONE	6	0.0117
221	06/30/98	42	Right	Control	Ethanol	HD	6	0.0226
222	06/30/98	42	Left	Control	None	NONE	6	0.0147
223	06/30/98	43	Right	Control	Ethanol	HD	6	0.0202
224	06/30/98	43	Left	Control	None	NONE	6	0.0158
225	06/30/98	44	Right	Control	Ethanol	HD	6	0.0271
226	06/30/98	44	Left	Control	None	NONE	6	0.0157
227	06/30/98	45	Right	Control	Ethanol	HD	6	0.0215
228	06/30/98	45	Left	Control	None	NONE	6	0.0143

SAP Module III SAS Dataset Created by DBMS Copy from the Microsoft Excel File
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Obs	DoseDate	Animal ID	Dose Site	Group	PreTreatment	Necropsy Exposure	Time	SAP
1	08/13/98	1	Right	Control	Ethanol	HD	6	10.2739
2	08/13/98	1	Left	Control	None	NONE	6	4.3521
3	08/13/98	2	Right	Control	Ethanol	HD	6	11.8677
4	08/13/98	2	Left	Control	None	NONE	6	6.5069
5	08/13/98	3	Right	Control	Ethanol	HD	6	3.4420
6	08/13/98	3	Left	Control	None	NONE	6	2.1092
7	08/13/98	4	Right	Control	Ethanol	HD	6	8.5653
8	08/13/98	4	Left	Control	None	NONE	6	2.7954
9	08/13/98	5	Right	Control	Ethanol	HD	6	21.9420
10	08/13/98	5	Left	Control	None	NONE	6	4.9711
11	08/13/98	6	Right	Control	Ethanol	HD	6	7.2655
12	08/13/98	6	Left	Control	None	NONE	6	1.9775
13	08/13/98	7	Right	Control	Ethanol	HD	6	6.2048
14	08/13/98	7	Left	Control	None	NONE	6	3.0554
15	08/13/98	8	Right	Control	Ethanol	HD	6	3.8887
16	08/13/98	8	Left	Control	None	NONE	6	1.3913
17	08/13/98	9	Right	Control	Ethanol	HD	6	6.8201
18	08/13/98	9	Left	Control	None	NONE	6	1.0668
19	08/13/98	10	Right	Control	Ethanol	HD	6	10.2753
20	08/13/98	10	Left	Control	None	NONE	6	3.5299
21	08/13/98	29	Right	Control	Ethanol	HD	24	0.2725
22	08/13/98	29	Left	Control	None	NONE	24	63.3961
23	08/13/98	30	Right	Control	Ethanol	HD	24	7.5359
24	08/13/98	30	Left	Control	None	NONE	24	10.9975
25	08/13/98	31	Right	Control	Ethanol	HD	24	4.7572
26	08/13/98	31	Left	Control	None	NONE	24	2.5972
27	08/13/98	32	Right	Control	Ethanol	HD	24	6.0026
28	08/13/98	32	Left	Control	None	NONE	24	5.6677
29	08/13/98	33	Right	Control	Ethanol	HD	24	4.8830
30	08/13/98	33	Left	Control	None	NONE	24	5.3249
31	08/13/98	34	Right	Control	Ethanol	HD	24	3.6917
32	08/13/98	34	Left	Control	None	NONE	24	2.7804
33	08/13/98	35	Right	Control	Ethanol	HD	24	4.9174
34	08/13/98	35	Left	Control	None	NONE	24	6.8494
35	08/13/98	36	Right	Control	Ethanol	HD	24	5.5619
36	08/13/98	36	Left	Control	None	NONE	24	3.8498
37	08/13/98	37	Right	Control	Ethanol	HD	24	8.8159
38	08/13/98	37	Left	Control	None	NONE	24	3.6841
39	08/13/98	38	Right	Control	Ethanol	HD	24	0.5276
40	08/13/98	38	Left	Control	None	NONE	24	5.5087
41	07/01/98	1	Right	Hydrocortisone	Hydrocortisone	HD	24	4.6217
42	07/01/98	1	Left	Hydrocortisone	None	NONE	24	1.7007
43	07/01/98	2	Right	Hydrocortisone	Hydrocortisone	HD	24	12.8326
44	07/01/98	2	Left	Hydrocortisone	None	NONE	24	13.7233
45	07/01/98	3	Right	Hydrocortisone	Hydrocortisone	HD	24	3.1408
46	07/01/98	3	Left	Hydrocortisone	None	NONE	24	1.8430
47	07/01/98	4	Right	Hydrocortisone	Hydrocortisone	HD	24	3.8703
48	07/01/98	4	Left	Hydrocortisone	None	NONE	24	1.2429
49	07/01/98	5	Right	Hydrocortisone	Hydrocortisone	HD	24	9.1992
50	07/01/98	5	Left	Hydrocortisone	None	NONE	24	1.2420
51	07/01/98	6	Right	Hydrocortisone	Hydrocortisone	HD	24	3.6738
52	07/01/98	6	Left	Hydrocortisone	None	NONE	24	2.4617
53	07/01/98	7	Right	Hydrocortisone	Hydrocortisone	HD	24	2.5932
54	07/01/98	7	Left	Hydrocortisone	None	NONE	24	1.2762
55	07/01/98	8	Right	Hydrocortisone	Hydrocortisone	HD	24	5.5253
56	07/01/98	8	Left	Hydrocortisone	None	NONE	24	1.9845
57	07/01/98	9	Right	Hydrocortisone	Hydrocortisone	HD	24	3.1704
58	07/01/98	9	Left	Hydrocortisone	None	NONE	24	1.3115
59	07/01/98	10	Right	Hydrocortisone	Hydrocortisone	HD	24	4.8100
60	07/01/98	10	Left	Hydrocortisone	None	NONE	24	1.4152
61	07/01/98	11	Right	Dexamethasone	Dexamethasone	HD	24	20.4670
62	07/01/98	11	Left	Dexamethasone	None	NONE	24	4.0090
63	07/01/98	12	Right	Dexamethasone	Dexamethasone	HD	24	7.2201
64	07/01/98	12	Left	Dexamethasone	None	NONE	24	2.1999
65	07/01/98	13	Right	Dexamethasone	Dexamethasone	HD	24	.
66	07/01/98	13	Left	Dexamethasone	None	NONE	24	.
67	07/01/98	14	Right	Dexamethasone	Dexamethasone	HD	24	3.3505
68	07/01/98	14	Left	Dexamethasone	None	NONE	24	1.7697
69	07/01/98	15	Right	Dexamethasone	Dexamethasone	HD	24	4.0327
70	07/01/98	15	Left	Dexamethasone	None	NONE	24	1.7357
71	07/01/98	16	Right	Dexamethasone	Dexamethasone	HD	24	.

MREF Task 41
SAP Module III SAS Dataset Created by DBMS Copy from the Microsoft Excel File
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Obs	DoseDate	Animal ID	Dose Site	Group	PreTreatment	Exposure	Necropsy Time	SAP
72	07/01/98	16	Left	Dexamethasone	None	NONE	24.	
73	07/01/98	17	Right	Dexamethasone	Dexamethasone	HD	24	2.8532
74	07/01/98	17	Left	Dexamethasone	None	NONE	24	1.1941
75	07/01/98	18	Right	Dexamethasone	Dexamethasone	HD	24	1.3139
76	07/01/98	18	Left	Dexamethasone	None	NONE	24	0.7203
77	07/01/98	19	Right	Dexamethasone	Dexamethasone	HD	24	3.2959
78	07/01/98	19	Left	Dexamethasone	None	NONE	24	1.5784
79	07/01/98	20	Right	Dexamethasone	Dexamethasone	HD	24	5.8927
80	07/01/98	20	Left	Dexamethasone	None	NONE	24	1.2960
81	07/01/98	21	Right	Olvanil	Olvanil	HD	24	4.6261
82	07/01/98	21	Left	Olvanil	None	NONE	24	1.6079
83	07/01/98	22	Right	Olvanil	Olvanil	HD	24	5.4328
84	07/01/98	22	Left	Olvanil	None	NONE	24	1.2153
85	07/01/98	23	Right	Olvanil	Olvanil	HD	24	2.2161
86	07/01/98	23	Left	Olvanil	None	NONE	24	1.6526
87	07/01/98	24	Right	Olvanil	Olvanil	HD	24	1.5464
88	07/01/98	24	Left	Olvanil	None	NONE	24	3.2243
89	07/01/98	25	Right	Olvanil	Olvanil	HD	24	3.7612
90	07/01/98	25	Left	Olvanil	None	NONE	24	0.7927
91	07/01/98	26	Right	Olvanil	Olvanil	HD	24	3.8431
92	07/01/98	26	Left	Olvanil	None	NONE	24	1.6098
93	07/01/98	27	Right	Olvanil	Olvanil	HD	24	2.9492
94	07/01/98	27	Left	Olvanil	None	NONE	24	1.5622
95	07/01/98	28	Right	Olvanil	Olvanil	HD	24	4.7420
96	07/01/98	28	Left	Olvanil	None	NONE	24	1.8491
97	07/01/98	29	Right	Olvanil	Olvanil	HD	24	34.2265
98	07/01/98	29	Left	Olvanil	None	NONE	24	4.7199
99	07/01/98	30	Right	Olvanil	Olvanil	HD	24	11.2880
100	07/01/98	30	Left	Olvanil	None	NONE	24	2.2540
101	07/01/98	31	Right	Indomethacin	Indomethacin	HD	24	67.4076
102	07/01/98	31	Left	Indomethacin	None	NONE	24	3.0251
103	07/01/98	32	Right	Indomethacin	Indomethacin	HD	24	8.0357
104	07/01/98	32	Left	Indomethacin	None	NONE	24	1.2571
105	07/01/98	33	Right	Indomethacin	Indomethacin	HD	24	24.6425
106	07/01/98	33	Left	Indomethacin	None	NONE	24	1.4226
107	07/01/98	34	Right	Indomethacin	Indomethacin	HD	24	36.8838
108	07/01/98	34	Left	Indomethacin	None	NONE	24	3.7833
109	07/01/98	35	Right	Indomethacin	Indomethacin	HD	24	71.5080
110	07/01/98	35	Left	Indomethacin	None	NONE	24	5.3808
111	07/01/98	36	Right	Indomethacin	Indomethacin	HD	24	55.0581
112	07/01/98	36	Left	Indomethacin	None	NONE	24	3.3537
113	07/01/98	37	Right	Indomethacin	Indomethacin	HD	24	53.5493
114	07/01/98	37	Left	Indomethacin	None	NONE	24	3.2463
115	07/01/98	38	Right	Indomethacin	Indomethacin	HD	24	16.7708
116	07/01/98	38	Left	Indomethacin	None	NONE	24	2.7812
117	07/01/98	39	Right	Indomethacin	Indomethacin	HD	24	30.5395
118	07/01/98	39	Left	Indomethacin	None	NONE	24	1.2290
119	07/01/98	40	Right	Indomethacin	Indomethacin	HD	24	19.0112
120	07/01/98	40	Left	Indomethacin	None	NONE	24	1.8945
121	07/01/98	41	Right	Control	Ethanol	HD	24	19.9402
122	07/01/98	41	Left	Control	None	NONE	24	2.1345
123	07/01/98	42	Right	Control	Ethanol	HD	24	23.2362
124	07/01/98	42	Left	Control	None	NONE	24	53.0821
125	07/01/98	43	Right	Control	Ethanol	HD	24	9.6486
126	07/01/98	43	Left	Control	None	NONE	24	2.2510
127	07/01/98	44	Right	Control	Ethanol	HD	24	11.4700
128	07/01/98	44	Left	Control	None	NONE	24	4.5502
129	07/01/98	45	Right	Control	Ethanol	HD	24	8.1517
130	07/01/98	45	Left	Control	None	NONE	24	4.1202
131	07/01/98	46	Right	Naive	None	NONE	24	3.1204
132	07/01/98	46	Left	Naive	None	NONE	24	3.4832
133	07/01/98	47	Right	Naive	None	NONE	24	6.0096
134	07/01/98	47	Left	Naive	None	NONE	24	4.3141
135	07/01/98	48	Right	Naive	None	NONE	24	2.7472
136	07/01/98	48	Left	Naive	None	NONE	24	3.3636
137	07/01/98	49	Right	Naive	None	NONE	24	2.5550
138	07/01/98	49	Left	Naive	None	NONE	24	3.1166
139	07/01/98	50	Right	Naive	None	NONE	24	3.5500
140	07/01/98	50	Left	Naive	None	NONE	24	2.9774
141	06/30/98	1	Right	Hydrocortisone	Hydrocortisone	HD	6	2.2097
142	06/30/98	1	Left	Hydrocortisone	None	NONE	6	3.7086

SAP Module III SAS Dataset Created by DBMS Copy from the Microsoft Excel File
SAP Data.1.xls - SAP

Obs	DoseDate	Animal ID	Dose Site	Group	PreTreatment	Exposure	Necropsy Time	SAP
143	06/30/98	2	Right	Hydrocortisone	Hydrocortisone	HD	6	10.4322
144	06/30/98	2	Left	Hydrocortisone	None	NONE	6	4.4588
145	06/30/98	3	Right	Hydrocortisone	Hydrocortisone	HD	6	3.6858
146	06/30/98	3	Left	Hydrocortisone	None	NONE	6	4.2624
147	06/30/98	4	Right	Hydrocortisone	Hydrocortisone	HD	6	3.2331
148	06/30/98	4	Left	Hydrocortisone	None	NONE	6	3.1639
149	06/30/98	5	Right	Hydrocortisone	Hydrocortisone	HD	6	2.4970
150	06/30/98	5	Left	Hydrocortisone	None	NONE	6	2.1302
151	06/30/98	6	Right	Hydrocortisone	Hydrocortisone	HD	6	1.6204
152	06/30/98	6	Left	Hydrocortisone	None	NONE	6	1.5897
153	06/30/98	7	Right	Hydrocortisone	Hydrocortisone	HD	6	2.7174
154	06/30/98	7	Left	Hydrocortisone	None	NONE	6	3.8382
155	06/30/98	8	Right	Hydrocortisone	Hydrocortisone	HD	6	1.5756
156	06/30/98	8	Left	Hydrocortisone	None	NONE	6	2.0593
157	06/30/98	9	Right	Hydrocortisone	Hydrocortisone	HD	6	2.7817
158	06/30/98	9	Left	Hydrocortisone	None	NONE	6	1.5423
159	06/30/98	10	Right	Hydrocortisone	Hydrocortisone	HD	6	2.6815
160	06/30/98	10	Left	Hydrocortisone	None	NONE	6	2.0197
161	06/30/98	11	Right	Dexamethasone	Dexamethasone	HD	6	5.2417
162	06/30/98	11	Left	Dexamethasone	None	NONE	6	4.6666
163	06/30/98	12	Right	Dexamethasone	Dexamethasone	HD	6	6.1189
164	06/30/98	12	Left	Dexamethasone	None	NONE	6	4.2452
165	06/30/98	13	Right	Dexamethasone	Dexamethasone	HD	6	1.5355
166	06/30/98	13	Left	Dexamethasone	None	NONE	6	0.6924
167	06/30/98	14	Right	Dexamethasone	Dexamethasone	HD	6	4.9169
168	06/30/98	14	Left	Dexamethasone	None	NONE	6	1.7565
169	06/30/98	15	Right	Dexamethasone	Dexamethasone	HD	6	3.6047
170	06/30/98	15	Left	Dexamethasone	None	NONE	6	3.0572
171	06/30/98	16	Right	Dexamethasone	Dexamethasone	HD	6	5.2402
172	06/30/98	16	Left	Dexamethasone	None	NONE	6	1.2818
173	06/30/98	17	Right	Dexamethasone	Dexamethasone	HD	6	3.7540
174	06/30/98	17	Left	Dexamethasone	None	NONE	6	2.5746
175	06/30/98	18	Right	Dexamethasone	Dexamethasone	HD	6	0.4719
176	06/30/98	18	Left	Dexamethasone	None	NONE	6	0.3662
177	06/30/98	19	Right	Dexamethasone	Dexamethasone	HD	6	1.1171
178	06/30/98	19	Left	Dexamethasone	None	NONE	6	0.5914
179	06/30/98	20	Right	Dexamethasone	Dexamethasone	HD	6	1.3157
180	06/30/98	20	Left	Dexamethasone	None	NONE	6	0.9768
181	06/30/98	21	Right	Olvanil	Olvanil	HD	6	2.1376
182	06/30/98	21	Left	Olvanil	None	NONE	6	0.6285
183	06/30/98	22	Right	Olvanil	Olvanil	HD	6	0.2721
184	06/30/98	22	Left	Olvanil	None	NONE	6	0.1924
185	06/30/98	23	Right	Olvanil	Olvanil	HD	6	1.1925
186	06/30/98	23	Left	Olvanil	None	NONE	6	0.6160
187	06/30/98	24	Right	Olvanil	Olvanil	HD	6	1.8369
188	06/30/98	24	Left	Olvanil	None	NONE	6	0.4960
189	06/30/98	25	Right	Olvanil	Olvanil	HD	6	1.4094
190	06/30/98	25	Left	Olvanil	None	NONE	6	0.5557
191	06/30/98	26	Right	Olvanil	Olvanil	HD	6	1.6248
192	06/30/98	26	Left	Olvanil	None	NONE	6	0.6253
193	06/30/98	27	Right	Olvanil	Olvanil	HD	6	4.6918
194	06/30/98	27	Left	Olvanil	None	NONE	6	4.6139
195	06/30/98	28	Right	Olvanil	Olvanil	HD	6	0.8713
196	06/30/98	28	Left	Olvanil	None	NONE	6	0.2721
197	06/30/98	29	Right	Olvanil	Olvanil	HD	6	4.7318
198	06/30/98	29	Left	Olvanil	None	NONE	6	3.1947
199	06/30/98	30	Right	Olvanil	Olvanil	HD	6	0.8190
200	06/30/98	30	Left	Olvanil	None	NONE	6	0.5632
201	06/30/98	31	Right	Indomethacin	Indomethacin	HD	6	1.8128
202	06/30/98	31	Left	Indomethacin	None	NONE	6	0.7564
203	06/30/98	32	Right	Indomethacin	Indomethacin	HD	6	2.7869
204	06/30/98	32	Left	Indomethacin	None	NONE	6	2.4336
205	06/30/98	33	Right	Indomethacin	Indomethacin	HD	6	2.0817
206	06/30/98	33	Left	Indomethacin	None	NONE	6	0.7475
207	06/30/98	34	Right	Indomethacin	Indomethacin	HD	6	0.8413
208	06/30/98	34	Left	Indomethacin	None	NONE	6	0.4566
209	06/30/98	35	Right	Indomethacin	Indomethacin	HD	6	2.8809
210	06/30/98	35	Left	Indomethacin	None	NONE	6	1.6186
211	06/30/98	37	Right	Indomethacin	Indomethacin	HD	6	5.4811
212	06/30/98	37	Left	Indomethacin	None	NONE	6	1.6751
213	06/30/98	38	Right	Indomethacin	Indomethacin	HD	6	2.2765

SAP Module III SAS Dataset Created by DBMS Copy from the Microsoft Excel File
SAP Data.1.xls - SAP

Obs	DoseDate	Animal ID	Dose Site	Group	PreTreatment	Exposure	Necropsy Time	SAP
214	06/30/98	38	Left	Indomethacin	None	NONE	6	0.64915
215	06/30/98	39	Right	Indomethacin	Indomethacin	HD	6	0.40580
216	06/30/98	39	Left	Indomethacin	None	NONE	6	0.48370
217	06/30/98	40	Right	Indomethacin	Indomethacin	HD	6	2.08644
218	06/30/98	40	Left	Indomethacin	None	NONE	6	2.53489
219	06/30/98	41	Right	Control	Ethanol	HD	6	1.30432
220	06/30/98	41	Left	Control	None	NONE	6	0.46361
221	06/30/98	42	Right	Control	Ethanol	HD	6	2.39622
222	06/30/98	42	Left	Control	None	NONE	6	0.58109
223	06/30/98	43	Right	Control	Ethanol	HD	6	0.36712
224	06/30/98	43	Left	Control	None	NONE	6	0.22655
225	06/30/98	44	Right	Control	Ethanol	HD	6	1.05256
226	06/30/98	44	Left	Control	None	NONE	6	0.35566
227	06/30/98	45	Right	Control	Ethanol	HD	6	1.40216
228	06/30/98	45	Left	Control	None	NONE	6	0.53136

IL-6 Module III SAS Dataset Created by DBMS Copy from the Microsoft Excel File
New IL-6 Corrected.xls - IL-6 Summary Mod III

Obs	Dosedate	Animal ID	Dose Site	Group	PreTreatment	Exposure	Necropsy Time	IL6
1	08/13/98	1	Right	Control	Ethanol	HD	6 Hours	-5.2386
2	08/13/98	1	Left	Control	None	NONE	6 Hours	-15.1280
3	08/13/98	2	Right	Control	Ethanol	HD	6 Hours	-5.2456
4	08/13/98	2	Left	Control	None	NONE	6 Hours	-18.1354
5	08/13/98	3	Right	Control	Ethanol	HD	6 Hours	-3.4805
6	08/13/98	3	Left	Control	None	NONE	6 Hours	-16.6536
7	08/13/98	4	Right	Control	Ethanol	HD	6 Hours	-2.1381
8	08/13/98	4	Left	Control	None	NONE	6 Hours	-11.6628
9	08/13/98	5	Right	Control	Ethanol	HD	6 Hours	-4.4962
10	08/13/98	5	Left	Control	None	NONE	6 Hours	-10.8685
11	08/13/98	6	Right	Control	Ethanol	HD	6 Hours	0.2541
12	08/13/98	6	Left	Control	None	NONE	6 Hours	-9.6522
13	08/13/98	7	Right	Control	Ethanol	HD	6 Hours	-2.9868
14	08/13/98	7	Left	Control	None	NONE	6 Hours	-9.1841
15	08/13/98	8	Right	Control	Ethanol	HD	6 Hours	-1.0804
16	08/13/98	8	Left	Control	None	NONE	6 Hours	-7.2206
17	08/13/98	9	Right	Control	Ethanol	HD	6 Hours	7.2597
18	08/13/98	9	Left	Control	None	NONE	6 Hours	-3.5221
19	08/13/98	10	Right	Control	Ethanol	HD	6 Hours	-6.1931
20	08/13/98	10	Left	Control	None	NONE	6 Hours	-11.5558
21	08/13/98	29	Right	Control	Ethanol	HD	24 Hours	-0.2821
22	08/13/98	29	Left	Control	None	NONE	24 Hours	-13.8017
23	08/13/98	30	Right	Control	Ethanol	HD	24 Hours	0.3078
24	08/13/98	30	Left	Control	None	NONE	24 Hours	-7.3681
25	08/13/98	31	Right	Control	Ethanol	HD	24 Hours	0.2971
26	08/13/98	31	Left	Control	None	NONE	24 Hours	-8.3311
27	08/13/98	32	Right	Control	Ethanol	HD	24 Hours	1.3551
28	08/13/98	32	Left	Control	None	NONE	24 Hours	-11.4877
29	08/13/98	33	Right	Control	Ethanol	HD	24 Hours	0.2666
30	08/13/98	33	Left	Control	None	NONE	24 Hours	-6.5495
31	08/13/98	34	Right	Control	Ethanol	HD	24 Hours	-0.4156
32	08/13/98	34	Left	Control	None	NONE	24 Hours	-4.6659
33	08/13/98	35	Right	Control	Ethanol	HD	24 Hours	0.8242
34	08/13/98	35	Left	Control	None	NONE	24 Hours	-7.8898
35	08/13/98	36	Right	Control	Ethanol	HD	24 Hours	0.2476
36	08/13/98	36	Left	Control	None	NONE	24 Hours	-6.2401
37	08/13/98	37	Right	Control	Ethanol	HD	24 Hours	1.5322
38	08/13/98	37	Left	Control	None	NONE	24 Hours	-6.1926
39	08/13/98	38	Right	Control	Ethanol	HD	24 Hours	0.1376
40	08/13/98	38	Left	Control	None	NONE	24 Hours	-6.1539
41	07/01/98	1	Right	Hydrocortisone	Hydrocortisone	HD	24 Hours	1.0987
42	07/01/98	1	Left	Hydrocortisone	None	NONE	24 Hours	-4.6015
43	07/01/98	2	Right	Hydrocortisone	Hydrocortisone	HD	24 Hours	-0.0458
44	07/01/98	2	Left	Hydrocortisone	None	NONE	24 Hours	-4.3378
45	07/01/98	3	Right	Hydrocortisone	Hydrocortisone	HD	24 Hours	0.2607
46	07/01/98	3	Left	Hydrocortisone	None	NONE	24 Hours	-4.1287
47	07/01/98	4	Right	Hydrocortisone	Hydrocortisone	HD	24 Hours	1.2927
48	07/01/98	4	Left	Hydrocortisone	None	NONE	24 Hours	-3.3741
49	07/01/98	5	Right	Hydrocortisone	Hydrocortisone	HD	24 Hours	0.2362
50	07/01/98	5	Left	Hydrocortisone	None	NONE	24 Hours	-3.8412
51	07/01/98	6	Right	Hydrocortisone	Hydrocortisone	HD	24 Hours	1.9165
52	07/01/98	6	Left	Hydrocortisone	None	NONE	24 Hours	-1.7168
53	07/01/98	7	Right	Hydrocortisone	Hydrocortisone	HD	24 Hours	0.7845
54	07/01/98	7	Left	Hydrocortisone	None	NONE	24 Hours	-3.3008
55	07/01/98	8	Right	Hydrocortisone	Hydrocortisone	HD	24 Hours	0.8827
56	07/01/98	8	Left	Hydrocortisone	None	NONE	24 Hours	-3.0334
57	07/01/98	9	Right	Hydrocortisone	Hydrocortisone	HD	24 Hours	0.5417
58	07/01/98	9	Left	Hydrocortisone	None	NONE	24 Hours	-2.6305
59	07/01/98	10	Right	Hydrocortisone	Hydrocortisone	HD	24 Hours	0.0348
60	07/01/98	10	Left	Hydrocortisone	None	NONE	24 Hours	-2.6873
61	07/01/98	11	Right	Dexamethasone	Dexamethasone	HD	24 Hours	-0.3139
62	07/01/98	11	Left	Dexamethasone	None	NONE	24 Hours	-6.0893
63	07/01/98	12	Right	Dexamethasone	Dexamethasone	HD	24 Hours	-0.1219
64	07/01/98	12	Left	Dexamethasone	None	NONE	24 Hours	-6.3988
65	07/01/98	13	Right	Dexamethasone	Dexamethasone	HD	24 Hours	-0.4244
66	07/01/98	13	Left	Dexamethasone	None	NONE	24 Hours	-2.9649
67	07/01/98	14	Right	Dexamethasone	Dexamethasone	HD	24 Hours	0.1642
68	07/01/98	14	Left	Dexamethasone	None	NONE	24 Hours	-3.3138
69	07/01/98	15	Right	Dexamethasone	Dexamethasone	HD	24 Hours	-0.0420
70	07/01/98	15	Left	Dexamethasone	None	NONE	24 Hours	-5.5752
71	07/01/98	16	Right	Dexamethasone	Dexamethasone	HD	24 Hours	-0.0787

IL-6 Module III SAS Dataset Created by DBMS Copy from the Microsoft Excel File New IL-6 Corrected.xls - IL-6 Summary Mod III

Obs	Dosedate	Animal ID	Dose Site	Group	PreTreatment	Exposure	Necropsy Time	IL6
72	07/01/98	16	Left	Dexamethasone	None	NONE	24 Hours	-3.6957
73	07/01/98	17	Right	Dexamethasone	Dexamethasone	HD	24 Hours	-0.0812
74	07/01/98	17	Left	Dexamethasone	None	NONE	24 Hours	-2.8249
75	07/01/98	18	Right	Dexamethasone	Dexamethasone	HD	24 Hours	-1.5051
76	07/01/98	18	Left	Dexamethasone	None	NONE	24 Hours	-11.5555
77	07/01/98	19	Right	Dexamethasone	Dexamethasone	HD	24 Hours	0.0806
78	07/01/98	19	Left	Dexamethasone	None	NONE	24 Hours	-4.4848
79	07/01/98	20	Right	Dexamethasone	Dexamethasone	HD	24 Hours	-2.0252
80	07/01/98	20	Left	Dexamethasone	None	NONE	24 Hours	-26.6207
81	07/01/98	21	Right	Olvanil	Olvanil	HD	24 Hours	-2.7183
82	07/01/98	21	Left	Olvanil	None	NONE	24 Hours	-19.6954
83	07/01/98	22	Right	Olvanil	Olvanil	HD	24 Hours	0.8802
84	07/01/98	22	Left	Olvanil	None	NONE	24 Hours	-8.6443
85	07/01/98	23	Right	Olvanil	Olvanil	HD	24 Hours	-0.2014
86	07/01/98	23	Left	Olvanil	None	NONE	24 Hours	-5.6378
87	07/01/98	24	Right	Olvanil	Olvanil	HD	24 Hours	-0.8914
88	07/01/98	24	Left	Olvanil	None	NONE	24 Hours	-8.6737
89	07/01/98	25	Right	Olvanil	Olvanil	HD	24 Hours	-0.7179
90	07/01/98	25	Left	Olvanil	None	NONE	24 Hours	-4.6674
91	07/01/98	26	Right	Olvanil	Olvanil	HD	24 Hours	-0.1966
92	07/01/98	26	Left	Olvanil	None	NONE	24 Hours	-7.8181
93	07/01/98	27	Right	Olvanil	Olvanil	HD	24 Hours	-0.3798
94	07/01/98	27	Left	Olvanil	None	NONE	24 Hours	-6.6218
95	07/01/98	28	Right	Olvanil	Olvanil	HD	24 Hours	-0.4773
96	07/01/98	28	Left	Olvanil	None	NONE	24 Hours	-6.3121
97	07/01/98	29	Right	Olvanil	Olvanil	HD	24 Hours	-0.3312
98	07/01/98	29	Left	Olvanil	None	NONE	24 Hours	-8.4984
99	07/01/98	30	Right	Olvanil	Olvanil	HD	24 Hours	0.8593
100	07/01/98	30	Left	Olvanil	None	NONE	24 Hours	-8.1031
101	07/01/98	31	Right	Indomethacin	Indomethacin	HD	24 Hours	-0.1155
102	07/01/98	31	Left	Indomethacin	None	NONE	24 Hours	-12.8656
103	07/01/98	32	Right	Indomethacin	Indomethacin	HD	24 Hours	-0.7681
104	07/01/98	32	Left	Indomethacin	None	NONE	24 Hours	-5.3838
105	07/01/98	33	Right	Indomethacin	Indomethacin	HD	24 Hours	0.1307
106	07/01/98	33	Left	Indomethacin	None	NONE	24 Hours	-5.7058
107	07/01/98	34	Right	Indomethacin	Indomethacin	HD	24 Hours	0.2297
108	07/01/98	34	Left	Indomethacin	None	NONE	24 Hours	-3.9645
109	07/01/98	35	Right	Indomethacin	Indomethacin	HD	24 Hours	-0.0505
110	07/01/98	35	Left	Indomethacin	None	NONE	24 Hours	-6.4663
111	07/01/98	36	Right	Indomethacin	Indomethacin	HD	24 Hours	0.0954
112	07/01/98	36	Left	Indomethacin	None	NONE	24 Hours	-7.2986
113	07/01/98	37	Right	Indomethacin	Indomethacin	HD	24 Hours	2.0056
114	07/01/98	37	Left	Indomethacin	None	NONE	24 Hours	-4.0168
115	07/01/98	38	Right	Indomethacin	Indomethacin	HD	24 Hours	1.9300
116	07/01/98	38	Left	Indomethacin	None	NONE	24 Hours	-8.5482
117	07/01/98	39	Right	Indomethacin	Indomethacin	HD	24 Hours	2.7251
118	07/01/98	39	Left	Indomethacin	None	NONE	24 Hours	-1.3568
119	07/01/98	40	Right	Indomethacin	Indomethacin	HD	24 Hours	2.6909
120	07/01/98	40	Left	Indomethacin	None	NONE	24 Hours	0.7781
121	07/01/98	41	Right	Control	Ethanol	HD	24 Hours	1.0818
122	07/01/98	41	Left	Control	None	NONE	24 Hours	1.9398
123	07/01/98	42	Right	Control	Ethanol	HD	24 Hours	1.1041
124	07/01/98	42	Left	Control	None	NONE	24 Hours	8.9242
125	07/01/98	43	Right	Control	Ethanol	HD	24 Hours	0.7214
126	07/01/98	43	Left	Control	None	NONE	24 Hours	1.4039
127	07/01/98	44	Right	Control	Ethanol	HD	24 Hours	0.3130
128	07/01/98	44	Left	Control	None	NONE	24 Hours	1.1597
129	07/01/98	45	Right	Control	Ethanol	HD	24 Hours	0.4055
130	07/01/98	45	Left	Control	None	NONE	24 Hours	1.9184
131	07/01/98	46	Right	Naive	None	HD	24 Hours	3.5148
132	07/01/98	46	Left	Naive	None	NONE	24 Hours	2.5629
133	07/01/98	47	Right	Naive	None	HD	24 Hours	1.6248
134	07/01/98	47	Left	Naive	None	NONE	24 Hours	1.9875
135	07/01/98	48	Right	Naive	None	HD	24 Hours	1.4187
136	07/01/98	48	Left	Naive	None	NONE	24 Hours	2.2094
137	07/01/98	49	Right	Naive	None	HD	24 Hours	2.3339
138	07/01/98	49	Left	Naive	None	NONE	24 Hours	2.1265
139	07/01/98	50	Right	Naive	None	HD	24 Hours	2.4428
140	07/01/98	50	Left	Naive	None	NONE	24 Hours	2.2230
141	06/30/98	1	Right	Hydrocortisone	Hydrocortisone	HD	6 Hours	1.4899
142	06/30/98	1	Left	Hydrocortisone	None	NONE	6 Hours	1.2924

IL-6 Module III SAS Dataset Created by DBMS Copy from the Microsoft Excel File
New IL-6 Corrected.xls - IL-6 Summary Mod III

Obs	Necropsy Dosedate	AnimalID	DoseSite	Group	PreTreatment		ExposureTime	IL6
143	06/30/98	2	Right	Hydrocortisone	Hydrocortisone	HD	6 Hours	7.9420
144	06/30/98	2	Left	Hydrocortisone	None	NONE	6 Hours	9.5960
145	06/30/98	3	Right	Hydrocortisone	Hydrocortisone	HD	6 Hours	18.1187
146	06/30/98	3	Left	Hydrocortisone	None	NONE	6 Hours	12.4345
147	06/30/98	4	Right	Hydrocortisone	Hydrocortisone	HD	6 Hours	12.0290
148	06/30/98	4	Left	Hydrocortisone	None	NONE	6 Hours	11.3122
149	06/30/98	5	Right	Hydrocortisone	Hydrocortisone	HD	6 Hours	12.2229
150	06/30/98	5	Left	Hydrocortisone	None	NONE	6 Hours	9.9299
151	06/30/98	6	Right	Hydrocortisone	Hydrocortisone	HD	6 Hours	9.5974
152	06/30/98	6	Left	Hydrocortisone	None	NONE	6 Hours	9.0495
153	06/30/98	7	Right	Hydrocortisone	Hydrocortisone	HD	6 Hours	5.3364
154	06/30/98	7	Left	Hydrocortisone	None	NONE	6 Hours	7.8772
155	06/30/98	8	Right	Hydrocortisone	Hydrocortisone	HD	6 Hours	9.9169
156	06/30/98	8	Left	Hydrocortisone	None	NONE	6 Hours	9.0863
157	06/30/98	9	Right	Hydrocortisone	Hydrocortisone	HD	6 Hours	12.1370
158	06/30/98	9	Left	Hydrocortisone	None	NONE	6 Hours	5.7358
159	06/30/98	10	Right	Hydrocortisone	Hydrocortisone	HD	6 Hours	7.2441
160	06/30/98	10	Left	Hydrocortisone	None	NONE	6 Hours	6.1313
161	06/30/98	11	Right	Dexamethasone	Dexamethasone	HD	6 Hours	7.0088
162	06/30/98	11	Left	Dexamethasone	None	NONE	6 Hours	10.0938
163	06/30/98	12	Right	Dexamethasone	Dexamethasone	HD	6 Hours	6.1214
164	06/30/98	12	Left	Dexamethasone	None	NONE	6 Hours	10.3696
165	06/30/98	13	Right	Dexamethasone	Dexamethasone	HD	6 Hours	7.0617
166	06/30/98	13	Left	Dexamethasone	None	NONE	6 Hours	6.3695
167	06/30/98	14	Right	Dexamethasone	Dexamethasone	HD	6 Hours	6.2862
168	06/30/98	14	Left	Dexamethasone	None	NONE	6 Hours	6.7349
169	06/30/98	15	Right	Dexamethasone	Dexamethasone	HD	6 Hours	7.0642
170	06/30/98	15	Left	Dexamethasone	None	NONE	6 Hours	10.7211
171	06/30/98	16	Right	Dexamethasone	Dexamethasone	HD	6 Hours	4.4582
172	06/30/98	16	Left	Dexamethasone	None	NONE	6 Hours	10.7057
173	06/30/98	17	Right	Dexamethasone	Dexamethasone	HD	6 Hours	13.1822
174	06/30/98	17	Left	Dexamethasone	None	NONE	6 Hours	7.4214
175	06/30/98	18	Right	Dexamethasone	Dexamethasone	HD	6 Hours	6.2828
176	06/30/98	18	Left	Dexamethasone	None	NONE	6 Hours	11.1604
177	06/30/98	19	Right	Dexamethasone	Dexamethasone	HD	6 Hours	10.1047
178	06/30/98	19	Left	Dexamethasone	None	NONE	6 Hours	10.9498
179	06/30/98	20	Right	Dexamethasone	Dexamethasone	HD	6 Hours	11.2575
180	06/30/98	20	Left	Dexamethasone	None	NONE	6 Hours	11.9154
181	06/30/98	21	Right	Olvanil	Olvanil	HD	6 Hours	6.8846
182	06/30/98	21	Left	Olvanil	None	NONE	6 Hours	3.6288
183	06/30/98	22	Right	Olvanil	Olvanil	HD	6 Hours	6.1070
184	06/30/98	22	Left	Olvanil	None	NONE	6 Hours	2.0120
185	06/30/98	23	Right	Olvanil	Olvanil	HD	6 Hours	10.2841
186	06/30/98	23	Left	Olvanil	None	NONE	6 Hours	2.1021
187	06/30/98	24	Right	Olvanil	Olvanil	HD	6 Hours	8.6288
188	06/30/98	24	Left	Olvanil	None	NONE	6 Hours	2.0929
189	06/30/98	25	Right	Olvanil	Olvanil	HD	6 Hours	3.7241
190	06/30/98	25	Left	Olvanil	None	NONE	6 Hours	1.4113
191	06/30/98	26	Right	Olvanil	Olvanil	HD	6 Hours	10.2162
192	06/30/98	26	Left	Olvanil	None	NONE	6 Hours	1.5297
193	06/30/98	27	Right	Olvanil	Olvanil	HD	6 Hours	7.5940
194	06/30/98	27	Left	Olvanil	None	NONE	6 Hours	4.6877
195	06/30/98	28	Right	Olvanil	Olvanil	HD	6 Hours	4.6913
196	06/30/98	28	Left	Olvanil	None	NONE	6 Hours	0.2709
197	06/30/98	29	Right	Olvanil	Olvanil	HD	6 Hours	7.5344
198	06/30/98	29	Left	Olvanil	None	NONE	6 Hours	2.5916
199	06/30/98	30	Right	Olvanil	Olvanil	HD	6 Hours	11.6865
200	06/30/98	30	Left	Olvanil	None	NONE	6 Hours	4.4309
201	06/30/98	31	Right	Indomethacin	Indomethacin	HD	6 Hours	3.3053
202	06/30/98	31	Left	Indomethacin	None	NONE	6 Hours	0.7694
203	06/30/98	32	Right	Indomethacin	Indomethacin	HD	6 Hours	1.0278
204	06/30/98	32	Left	Indomethacin	None	NONE	6 Hours	3.0272
205	06/30/98	33	Right	Indomethacin	Indomethacin	HD	6 Hours	4.6212
206	06/30/98	33	Left	Indomethacin	None	NONE	6 Hours	1.2786
207	06/30/98	34	Right	Indomethacin	Indomethacin	HD	6 Hours	3.3046
208	06/30/98	34	Left	Indomethacin	None	NONE	6 Hours	1.2590
209	06/30/98	35	Right	Indomethacin	Indomethacin	HD	6 Hours	2.7907
210	06/30/98	35	Left	Indomethacin	None	NONE	6 Hours	1.3372
211	06/30/98	37	Right	Indomethacin	Indomethacin	HD	6 Hours	0.6773
212	06/30/98	37	Left	Indomethacin	None	NONE	6 Hours	1.9281
213	06/30/98	38	Right	Indomethacin	Indomethacin	HD	6 Hours	2.5045

IL-6 Module III SAS Dataset Created by DBMS Copy from the Microsoft Excel File
New IL-6 Corrected.xls - IL-6 Summary Mod III

Obs	Dosedate	Animal ID	Dose Site	Group	PreTreatment	Exposure	Necropsy Time	IL6
214	06/30/98	38	Left	Indomethacin	None	NONE	6 Hours	1.17131
215	06/30/98	39	Right	Indomethacin	Indomethacin	HD	6 Hours	2.47696
216	06/30/98	39	Left	Indomethacin	None	NONE	6 Hours	1.44412
217	06/30/98	40	Right	Indomethacin	Indomethacin	HD	6 Hours	2.87054
218	06/30/98	40	Left	Indomethacin	None	NONE	6 Hours	2.38026
219	06/30/98	41	Right	Control	Ethanol	HD	6 Hours	0.79521
220	06/30/98	41	Left	Control	None	NONE	6 Hours	0.42513
221	06/30/98	42	Right	Control	Ethanol	HD	6 Hours	0.93228
222	06/30/98	42	Left	Control	None	NONE	6 Hours	1.39577
223	06/30/98	43	Right	Control	Ethanol	HD	6 Hours	2.35781
224	06/30/98	43	Left	Control	None	NONE	6 Hours	1.27188
225	06/30/98	44	Right	Control	Ethanol	HD	6 Hours	0.88800
226	06/30/98	44	Left	Control	None	NONE	6 Hours	1.41254
227	06/30/98	45	Right	Control	Ethanol	HD	6 Hours	0.92828
228	06/30/98	45	Left	Control	None	NONE	6 Hours	5.89743

MPX Module III SAS Dataset Created by DBMS Copy from the Microsoft Excel File
MPX ModuleIII.1.xls - 063098

Obs	DoseDate	Animal ID	Dose Site	Group	PreTreatment	Exposure	Necropsy Time	MPX
1	06/30/98	1	Right	Hydrocortisone	Hydrocortisone	HD	6 Hours	1.26211
2	06/30/98	1	Left	Hydrocortisone	None	NONE	6 Hours	0.57894
3	06/30/98	2	Right	Hydrocortisone	Hydrocortisone	HD	6 Hours	0.24449
4	06/30/98	2	Left	Hydrocortisone	None	NONE	6 Hours	0.20701
5	06/30/98	3	Right	Hydrocortisone	Hydrocortisone	HD	6 Hours	0.23382
6	06/30/98	3	Left	Hydrocortisone	None	NONE	6 Hours	0.19576
7	06/30/98	4	Right	Hydrocortisone	Hydrocortisone	HD	6 Hours	1.11552
8	06/30/98	4	Left	Hydrocortisone	None	NONE	6 Hours	0.50235
9	06/30/98	5	Right	Hydrocortisone	Hydrocortisone	HD	6 Hours	1.11808
10	06/30/98	5	Left	Hydrocortisone	None	NONE	6 Hours	0.36402
11	06/30/98	6	Right	Hydrocortisone	Hydrocortisone	HD	6 Hours	1.75903
12	06/30/98	6	Left	Hydrocortisone	None	NONE	6 Hours	0.27604
13	06/30/98	7	Right	Hydrocortisone	Hydrocortisone	HD	6 Hours	2.37060
14	06/30/98	7	Left	Hydrocortisone	None	NONE	6 Hours	1.06543
15	06/30/98	8	Right	Hydrocortisone	Hydrocortisone	HD	6 Hours	1.54509
16	06/30/98	8	Left	Hydrocortisone	None	NONE	6 Hours	0.50680
17	06/30/98	9	Right	Hydrocortisone	Hydrocortisone	HD	6 Hours	1.71218
18	06/30/98	9	Left	Hydrocortisone	None	NONE	6 Hours	0.30014
19	06/30/98	10	Right	Hydrocortisone	Hydrocortisone	HD	6 Hours	1.15256
20	06/30/98	10	Left	Hydrocortisone	None	NONE	6 Hours	0.17303
21	06/30/98	11	Right	Dexamethasone	Dexamethasone	HD	6 Hours	1.20278
22	06/30/98	11	Left	Dexamethasone	None	NONE	6 Hours	0.27049
23	06/30/98	12	Right	Dexamethasone	Dexamethasone	HD	6 Hours	0.80958
24	06/30/98	12	Left	Dexamethasone	None	NONE	6 Hours	0.23912
25	06/30/98	13	Right	Dexamethasone	Dexamethasone	HD	6 Hours	0.91337
26	06/30/98	13	Left	Dexamethasone	None	NONE	6 Hours	0.35111
27	06/30/98	14	Right	Dexamethasone	Dexamethasone	HD	6 Hours	0.59398
28	06/30/98	14	Left	Dexamethasone	None	NONE	6 Hours	0.51501
29	06/30/98	15	Right	Dexamethasone	Dexamethasone	HD	6 Hours	1.47052
30	06/30/98	15	Left	Dexamethasone	None	NONE	6 Hours	0.80036
31	06/30/98	16	Right	Dexamethasone	Dexamethasone	HD	6 Hours	0.55959
32	06/30/98	16	Left	Dexamethasone	None	NONE	6 Hours	0.43480
33	06/30/98	17	Right	Dexamethasone	Dexamethasone	HD	6 Hours	0.67918
34	06/30/98	17	Left	Dexamethasone	None	NONE	6 Hours	0.56799
35	06/30/98	18	Right	Dexamethasone	Dexamethasone	HD	6 Hours	1.26718
36	06/30/98	18	Left	Dexamethasone	None	NONE	6 Hours	0.77527
37	06/30/98	19	Right	Dexamethasone	Dexamethasone	HD	6 Hours	0.80581
38	06/30/98	19	Left	Dexamethasone	None	NONE	6 Hours	0.38638
39	06/30/98	20	Right	Dexamethasone	Dexamethasone	HD	6 Hours	1.06328
40	06/30/98	20	Left	Dexamethasone	None	NONE	6 Hours	1.63423
41	06/30/98	21	Right	Olvanil	Olvanil	HD	6 Hours	0.76940
42	06/30/98	21	Left	Olvanil	None	NONE	6 Hours	0.98874
43	06/30/98	22	Right	Olvanil	Olvanil	HD	6 Hours	1.76640
44	06/30/98	22	Left	Olvanil	None	NONE	6 Hours	2.33469
45	06/30/98	23	Right	Olvanil	Olvanil	HD	6 Hours	1.70503
46	06/30/98	23	Left	Olvanil	None	NONE	6 Hours	0.48948
47	06/30/98	24	Right	Olvanil	Olvanil	HD	6 Hours	1.74915
48	06/30/98	24	Left	Olvanil	None	NONE	6 Hours	1.09465
49	06/30/98	25	Right	Olvanil	Olvanil	HD	6 Hours	1.38338
50	06/30/98	25	Left	Olvanil	None	NONE	6 Hours	0.67105
51	06/30/98	26	Right	Olvanil	Olvanil	HD	6 Hours	0.83695
52	06/30/98	26	Left	Olvanil	None	NONE	6 Hours	0.41528
53	06/30/98	27	Right	Olvanil	Olvanil	HD	6 Hours	1.66513
54	06/30/98	27	Left	Olvanil	None	NONE	6 Hours	0.54184
55	06/30/98	28	Right	Olvanil	Olvanil	HD	6 Hours	1.19505
56	06/30/98	28	Left	Olvanil	None	NONE	6 Hours	0.69941
57	06/30/98	29	Right	Olvanil	Olvanil	HD	6 Hours	1.98948
58	06/30/98	29	Left	Olvanil	None	NONE	6 Hours	0.55571
59	06/30/98	30	Right	Olvanil	Olvanil	HD	6 Hours	2.43404
60	06/30/98	30	Left	Olvanil	None	NONE	6 Hours	0.50455
61	06/30/98	31	Right	Indomethacin	Indomethacin	HD	6 Hours	2.95146
62	06/30/98	31	Left	Indomethacin	None	NONE	6 Hours	0.47425
63	06/30/98	32	Right	Indomethacin	Indomethacin	HD	6 Hours	1.62722
64	06/30/98	32	Left	Indomethacin	None	NONE	6 Hours	1.93469
65	06/30/98	33	Right	Indomethacin	Indomethacin	HD	6 Hours	1.73874
66	06/30/98	33	Left	Indomethacin	None	NONE	6 Hours	0.53882
67	06/30/98	34	Right	Indomethacin	Indomethacin	HD	6 Hours	0.79870
68	06/30/98	34	Left	Indomethacin	None	NONE	6 Hours	0.26390
69	06/30/98	35	Right	Indomethacin	Indomethacin	HD	6 Hours	1.36187
70	06/30/98	35	Left	Indomethacin	None	NONE	6 Hours	0.94972
71	06/30/98	36	Right	Indomethacin	Indomethacin	HD	6 Hours	.

MPX Module III SAS Dataset Created by DBMS Copy from the Microsoft Excel File
MPX ModuleIII.1.xls - 063098

Obs	DoseDate	Animal ID	Dose Site	Group	PreTreatment	Exposure	Necropsy Time	MPX
72	06/30/98	36	Left	Indomethacin	None	NONE	6 Hours	.
73	06/30/98	37	Right	Indomethacin	Indomethacin	HD	6 Hours	0.72027
74	06/30/98	37	Left	Indomethacin	None	NONE	6 Hours	0.52907
75	06/30/98	38	Right	Indomethacin	Indomethacin	HD	6 Hours	2.24581
76	06/30/98	38	Left	Indomethacin	None	NONE	6 Hours	0.33669
77	06/30/98	39	Right	Indomethacin	Indomethacin	HD	6 Hours	1.89094
78	06/30/98	39	Left	Indomethacin	None	NONE	6 Hours	0.41126
79	06/30/98	40	Right	Indomethacin	Indomethacin	HD	6 Hours	1.05914
80	06/30/98	40	Left	Indomethacin	None	NONE	6 Hours	0.23772
81	06/30/98	41	Right	Control	Ethanol	HD	6 Hours	0.88660
82	06/30/98	41	Left	Control	None	NONE	6 Hours	0.95675
83	06/30/98	42	Right	Control	Ethanol	HD	6 Hours	0.85189
84	06/30/98	42	Left	Control	None	NONE	6 Hours	0.32694
85	06/30/98	43	Right	Control	Ethanol	HD	6 Hours	0.81604
86	06/30/98	43	Left	Control	None	NONE	6 Hours	0.27540
87	06/30/98	44	Right	Control	Ethanol	HD	6 Hours	0.54283
88	06/30/98	44	Left	Control	None	NONE	6 Hours	0.44144
89	06/30/98	45	Right	Control	Ethanol	HD	6 Hours	0.74964
90	06/30/98	45	Left	Control	None	NONE	6 Hours	0.29385

MPX Module III SAS Dataset Created by DBMS Copy from the Microsoft Excel File
MPX ModuleIII.1.xls - 070198

Obs	DoseDate	Animal ID	Dose Site	Necropsy Group	PreTreatment	Exposure	Time	MPX
1	07/01/98	1	Right	Hydrocortisone	Hydrocortisone	HD	24 Hours	2.09765
2	07/01/98	1	Left	Hydrocortisone	None	NONE	24 Hours	0.18692
3	07/01/98	2	Right	Hydrocortisone	Hydrocortisone	HD	24 Hours	1.08918
4	07/01/98	2	Left	Hydrocortisone	None	NONE	24 Hours	0.23526
5	07/01/98	3	Right	Hydrocortisone	Hydrocortisone	HD	24 Hours	1.37137
6	07/01/98	3	Left	Hydrocortisone	None	NONE	24 Hours	0.65990
7	07/01/98	4	Right	Hydrocortisone	Hydrocortisone	HD	24 Hours	2.11661
8	07/01/98	4	Left	Hydrocortisone	None	NONE	24 Hours	0.22451
9	07/01/98	5	Right	Hydrocortisone	Hydrocortisone	HD	24 Hours	1.13377
10	07/01/98	5	Left	Hydrocortisone	None	NONE	24 Hours	0.38724
11	07/01/98	6	Right	Hydrocortisone	Hydrocortisone	HD	24 Hours	2.70821
12	07/01/98	6	Left	Hydrocortisone	None	NONE	24 Hours	0.31913
13	07/01/98	7	Right	Hydrocortisone	Hydrocortisone	HD	24 Hours	2.68906
14	07/01/98	7	Left	Hydrocortisone	None	NONE	24 Hours	0.41166
15	07/01/98	8	Right	Hydrocortisone	Hydrocortisone	HD	24 Hours	1.65584
16	07/01/98	8	Left	Hydrocortisone	None	NONE	24 Hours	0.53260
17	07/01/98	9	Right	Hydrocortisone	Hydrocortisone	HD	24 Hours	1.44163
18	07/01/98	9	Left	Hydrocortisone	None	NONE	24 Hours	0.38315
19	07/01/98	10	Right	Hydrocortisone	Hydrocortisone	HD	24 Hours	0.93634
20	07/01/98	10	Left	Hydrocortisone	None	NONE	24 Hours	0.28018
21	07/01/98	11	Right	Dexamethasone	Dexamethasone	HD	24 Hours	1.66732
22	07/01/98	11	Left	Dexamethasone	None	NONE	24 Hours	0.46435
23	07/01/98	12	Right	Dexamethasone	Dexamethasone	HD	24 Hours	1.60079
24	07/01/98	12	Left	Dexamethasone	None	NONE	24 Hours	0.20616
25	07/01/98	13	Right	Dexamethasone	Dexamethasone	HD	24 Hours	0.69854
26	07/01/98	13	Left	Dexamethasone	None	NONE	24 Hours	0.17859
27	07/01/98	14	Right	Dexamethasone	Dexamethasone	HD	24 Hours	1.43131
28	07/01/98	14	Left	Dexamethasone	None	NONE	24 Hours	0.21263
29	07/01/98	15	Right	Dexamethasone	Dexamethasone	HD	24 Hours	0.67920
30	07/01/98	15	Left	Dexamethasone	None	NONE	24 Hours	0.00400
31	07/01/98	16	Right	Dexamethasone	Dexamethasone	HD	24 Hours	1.99552
32	07/01/98	16	Left	Dexamethasone	None	NONE	24 Hours	0.14336
33	07/01/98	17	Right	Dexamethasone	Dexamethasone	HD	24 Hours	0.66462
34	07/01/98	17	Left	Dexamethasone	None	NONE	24 Hours	0.16341
35	07/01/98	18	Right	Dexamethasone	Dexamethasone	HD	24 Hours	1.47867
36	07/01/98	18	Left	Dexamethasone	None	NONE	24 Hours	0.10066
37	07/01/98	19	Right	Dexamethasone	Dexamethasone	HD	24 Hours	1.02353
38	07/01/98	19	Left	Dexamethasone	None	NONE	24 Hours	0.14757
39	07/01/98	20	Right	Dexamethasone	Dexamethasone	HD	24 Hours	1.02988
40	07/01/98	20	Left	Dexamethasone	None	NONE	24 Hours	0.07125
41	07/01/98	21	Right	Olvanil	Olvanil	HD	24 Hours	1.88737
42	07/01/98	21	Left	Olvanil	None	NONE	24 Hours	0.87710
43	07/01/98	22	Right	Olvanil	Olvanil	HD	24 Hours	1.32658
44	07/01/98	22	Left	Olvanil	None	NONE	24 Hours	0.54210
45	07/01/98	23	Right	Olvanil	Olvanil	HD	24 Hours	1.41614
46	07/01/98	23	Left	Olvanil	None	NONE	24 Hours	0.34752
47	07/01/98	24	Right	Olvanil	Olvanil	HD	24 Hours	2.82876
48	07/01/98	24	Left	Olvanil	None	NONE	24 Hours	0.58410
49	07/01/98	25	Right	Olvanil	Olvanil	HD	24 Hours	2.22309
50	07/01/98	25	Left	Olvanil	None	NONE	24 Hours	0.31730
51	07/01/98	26	Right	Olvanil	Olvanil	HD	24 Hours	1.76598
52	07/01/98	26	Left	Olvanil	None	NONE	24 Hours	0.20866
53	07/01/98	27	Right	Olvanil	Olvanil	HD	24 Hours	1.45302
54	07/01/98	27	Left	Olvanil	None	NONE	24 Hours	0.24593
55	07/01/98	28	Right	Olvanil	Olvanil	HD	24 Hours	3.19041
56	07/01/98	28	Left	Olvanil	None	NONE	24 Hours	0.46074
57	07/01/98	29	Right	Olvanil	Olvanil	HD	24 Hours	1.06350
58	07/01/98	29	Left	Olvanil	None	NONE	24 Hours	0.39297
59	07/01/98	30	Right	Olvanil	Olvanil	HD	24 Hours	1.49944
60	07/01/98	30	Left	Olvanil	None	NONE	24 Hours	1.80023
61	07/01/98	31	Right	Indomethacin	Indomethacin	HD	24 Hours	1.39054
62	07/01/98	31	Left	Indomethacin	None	NONE	24 Hours	0.67549
63	07/01/98	32	Right	Indomethacin	Indomethacin	HD	24 Hours	1.05996
64	07/01/98	32	Left	Indomethacin	None	NONE	24 Hours	0.83925
65	07/01/98	33	Right	Indomethacin	Indomethacin	HD	24 Hours	1.13692
66	07/01/98	33	Left	Indomethacin	None	NONE	24 Hours	0.20144
67	07/01/98	34	Right	Indomethacin	Indomethacin	HD	24 Hours	1.21121
68	07/01/98	34	Left	Indomethacin	None	NONE	24 Hours	0.61083
69	07/01/98	35	Right	Indomethacin	Indomethacin	HD	24 Hours	2.64154
70	07/01/98	35	Left	Indomethacin	None	NONE	24 Hours	0.16798
71	07/01/98	36	Right	Indomethacin	Indomethacin	HD	24 Hours	0.78676

MPX Module III SAS Dataset Created by DBMS Copy from the Microsoft Excel File
MPX ModuleIII.1.xls - 070198

Obs	DoseDate	Animal ID	Dose Site	Group	PreTreatment	Exposure	Necropsy Time	MPX
72	07/01/98	36	Left	Indomethacin	None	NONE	24 Hours	0.43685
73	07/01/98	37	Right	Indomethacin	Indomethacin	HD	24 Hours	1.00677
74	07/01/98	37	Left	Indomethacin	None	NONE	24 Hours	0.58983
75	07/01/98	38	Right	Indomethacin	Indomethacin	HD	24 Hours	2.97155
76	07/01/98	38	Left	Indomethacin	None	NONE	24 Hours	0.42938
77	07/01/98	39	Right	Indomethacin	Indomethacin	HD	24 Hours	3.18333
78	07/01/98	39	Left	Indomethacin	None	NONE	24 Hours	0.49196
79	07/01/98	40	Right	Indomethacin	Indomethacin	HD	24 Hours	2.71491
80	07/01/98	40	Left	Indomethacin	None	NONE	24 Hours	0.52086
81	07/01/98	41	Right	Control	Ethanol	HD	24 Hours	1.21841
82	07/01/98	41	Left	Control	None	NONE	24 Hours	0.69648
83	07/01/98	42	Right	Control	Ethanol	HD	24 Hours	1.47569
84	07/01/98	42	Left	Control	None	NONE	24 Hours	9.75550
85	07/01/98	43	Right	Control	Ethanol	HD	24 Hours	1.01690
86	07/01/98	43	Left	Control	None	NONE	24 Hours	0.17694
87	07/01/98	44	Right	Control	Ethanol	HD	24 Hours	0.69564
88	07/01/98	44	Left	Control	None	NONE	24 Hours	0.20667
89	07/01/98	45	Right	Control	Ethanol	HD	24 Hours	1.39177
90	07/01/98	45	Left	Control	None	NONE	24 Hours	0.42116
91	07/01/98	46	Right	Naive	None	NONE	24 Hours	0.70447
92	07/01/98	46	Left	Naive	None	NONE	24 Hours	0.65383
93	07/01/98	47	Right	Naive	None	NONE	24 Hours	0.16854
94	07/01/98	47	Left	Naive	None	NONE	24 Hours	0.14470
95	07/01/98	48	Right	Naive	None	NONE	24 Hours	0.40388
96	07/01/98	48	Left	Naive	None	NONE	24 Hours	0.28798
97	07/01/98	49	Right	Naive	None	NONE	24 Hours	1.58345
98	07/01/98	49	Left	Naive	None	NONE	24 Hours	0.81470
99	07/01/98	50	Right	Naive	None	NONE	24 Hours	0.90049
100	07/01/98	50	Left	Naive	None	NONE	24 Hours	1.26482

MPX Module III SAS Dataset Created by DBMS Copy from the Microsoft Excel File
MPX ModuleIII.1.xls - 081398

Obs	Animal dosedate	Dose Pre Necropsy ID	Site	Group	Treatment	Exposure	Time	MPX
1	08/13/98	1	Right	Control	Ethanol	HD	6 Hours	1.83829
2	08/13/98	1	Left	Control	None	NONE	6 Hours	0.34383
3	08/13/98	2	Right	Control	Ethanol	HD	6 Hours	0.98582
4	08/13/98	2	Left	Control	None	NONE	6 Hours	0.44831
5	08/13/98	3	Right	Control	Ethanol	HD	6 Hours	1.47482
6	08/13/98	3	Left	Control	None	NONE	6 Hours	0.49109
7	08/13/98	4	Right	Control	Ethanol	HD	6 Hours	1.51599
8	08/13/98	4	Left	Control	None	NONE	6 Hours	0.26039
9	08/13/98	5	Right	Control	Ethanol	HD	6 Hours	1.10030
10	08/13/98	5	Left	Control	None	NONE	6 Hours	0.25582
11	08/13/98	6	Right	Control	Ethanol	HD	6 Hours	1.08820
12	08/13/98	6	Left	Control	None	NONE	6 Hours	0.24190
13	08/13/98	7	Right	Control	Ethanol	HD	6 Hours	1.39549
14	08/13/98	7	Left	Control	None	NONE	6 Hours	0.53393
15	08/13/98	8	Right	Control	Ethanol	HD	6 Hours	2.29486
16	08/13/98	8	Left	Control	None	NONE	6 Hours	1.37407
17	08/13/98	9	Right	Control	Ethanol	HD	6 Hours	3.16360
18	08/13/98	9	Left	Control	None	NONE	6 Hours	0.57991
19	08/13/98	10	Right	Control	Ethanol	HD	6 Hours	1.54425
20	08/13/98	10	Left	Control	None	NONE	6 Hours	1.20994
21	08/13/98	29	Right	Control	Ethanol	HD	24 Hours	1.89779
22	08/13/98	29	Left	Control	None	NONE	24 Hours	0.21633
23	08/13/98	30	Right	Control	Ethanol	HD	24 Hours	11.82945
24	08/13/98	30	Left	Control	None	NONE	24 Hours	0.24494
25	08/13/98	31	Right	Control	Ethanol	HD	24 Hours	12.75012
26	08/13/98	31	Left	Control	None	NONE	24 Hours	0.40642
27	08/13/98	32	Right	Control	Ethanol	HD	24 Hours	13.30779
28	08/13/98	32	Left	Control	None	NONE	24 Hours	0.56877
29	08/13/98	33	Right	Control	Ethanol	HD	24 Hours	11.73687
30	08/13/98	33	Left	Control	None	NONE	24 Hours	0.08217
31	08/13/98	34	Right	Control	Ethanol	HD	24 Hours	10.70483
32	08/13/98	34	Left	Control	None	NONE	24 Hours	0.29682
33	08/13/98	35	Right	Control	Ethanol	HD	24 Hours	13.49886
34	08/13/98	35	Left	Control	None	NONE	24 Hours	0.89552
35	08/13/98	36	Right	Control	Ethanol	HD	24 Hours	11.62549
36	08/13/98	36	Left	Control	None	NONE	24 Hours	0.24931
37	08/13/98	37	Right	Control	Ethanol	HD	24 Hours	11.91410
38	08/13/98	37	Left	Control	None	NONE	24 Hours	0.46288
39	08/13/98	38	Right	Control	Ethanol	HD	24 Hours	10.97484
40	08/13/98	38	Left	Control	None	NONE	24 Hours	0.32258

APPENDIX J

Module II Histopathology Statistical Analyses

Battelle
. . . Putting Technology To Work

Project Number G1555-B41ASTAT (3104)

Internal Distribution

Lee/Dept. Files
NA Niemuth
JR Holdcraft
BJ Wood
RMO

s:\niem\mref\Task 41\Histopath_Mod2_report-Task 41 – final.doc

Date July 21, 2000

To **Michele Danne**

From Nancy Niemuth

Subject **Statistical Analysis of MREF Task 95-41 Module II Histopath Data - Revised**

The attached report summarizes the statistical analysis of histopathology and tissue weight data collected in Module II of MREF Task 95-41. The report has been revised to include comparisons of HD-exposed and sham sites within treatment groups (Table 4 and associated text). In addition, the appendix data listing has been included in the electronic file. An electronic copy of the MS-Word document that contains the report will be sent via electronic mail, for use in preparing the final report on this study.

Please call me at 424-3231 if you have any questions on the attached report.

NAN:llj
Attachment

For Review and Approval

	Name	Initials	Date
Originator	Nancy Niemuth	N	7/24/00
Concurrence	Jennifer Holdcraft	BJW	7/21/00
Approved	Bill Rosebrough	WRR	7/24/00

Sent: Interoffice mail

Statistical Report for MREF Task 95-41 Module II Histopath Data

July 21, 2000

Introduction

Experiments were conducted under MREF Task 95-41 Module II to determine the efficacy of four drug treatments: ICD 2086 (Indomethacin or IND), ICD 2723 (Olvanil or OLV), ICD 2842 (Hydrocortisone or HC), and ICD 2845 (Dexamethasone or DEX), in moderating the effect of HD exposure in the euthymic hairless mouse model. Four exposure sites were available on the back of each animal. Sites A and D were treated (IND, OLV, HC, DEX, or none) and exposed to HD vapor for 6 minutes; Sites B and C were sham sites. There were 10 animals in each drug treatment group and 8 control animals that received no drug treatment. The endpoints evaluated were tissue weight, and the histopath markers: microvesication, epidermal necrosis, pustular epidermitis, follicular necrosis, and intracellular edema.

Methods

The number of observations, mean and standard deviation of the severity score, and percent incidence in each drug treatment group (including the control group) were calculated for the histopath markers using the SAS (V8) MEANS procedure. Each exposure site was considered independently for this analysis, so there were 20 total observations for each drug treatment group (2 HD exposed sites on each of 10 animals) and 16 observations for the HD-exposed control group (2 HD exposed sites on each of 8 animals). The SAS (V8) FREQ procedure was used to perform Fisher's Exact Test for differences between each of the four drug treatments and the HD-exposed control group in the incidence of microvesication, epidermal necrosis, pustular epidermitis, follicular necrosis, and intracellular edema. The sham sites were not included in this analysis.

For tissue weights, the average response of the two drug treated/HD-exposed sites or two sham sites within each animal was used as the endpoint for statistical analysis. The statistical methods for the analysis of tissue weights were consistent with those used in the analysis of other quantitative endpoints collected in this study.

A two-stage approach was used to analyze the data for each endpoint, which entailed fitting analysis of variance (ANOVA) models to appropriate subsets of the data. The first model (Model 1) was fitted to data from the sham sites. Model 1 tested for a systemic effect due to drug treatment, for each drug treatment compound compared to the control. Based on the results of Model 1 analysis, either Model 2 or Model 3 was selected for the second stage.

If no significant differences were found in Model 1 (indicating there was no systemic effect due to either drug treatment compound) then Model 2 was implemented. Model 2 was

fitted to data from the drug treated/HD-exposed sites only. Model 2 tested for differences between HD-exposed sites on drug treated and control animals.

If significant differences were found between sham site responses for either drug treatment compound compared to control, indicating a systemic effect due to a drug treatment compound, Model 3 was employed. Model 3 tested for differences between treatment and control animals after adjusting for differences in sham site responses. For each animal, the response variable for Model 3 was calculated as the difference between the drug treated/HD-exposed site mean and sham site mean.

The ANOVA Models 1, 2, and 3 fitted to the tissue weight, SAP, IL-6, and MPX Module II data took the following form:

$$Y_{ij} = \mu + \beta_i + \varepsilon_{ij},$$

where Y_{ij} = tissue weight, SAP, IL-6, or MPX response for j^{th} animal receiving i^{th} treatment
μ = overall average value of the response
β_i = effect of i^{th} treatment
ε = uncontrolled variation.

The ANOVA models were fitted using the SAS (V8) MIXED procedure. For each endpoint, Dunnett's multiple comparison procedure was used to compare each drug treatment group to control. These comparisons are summarized for each model, as follows:

- Model 1: $SHAM_{trt} - SHAM_{ctl}$
- Model 2: $HD_{trt} - HD_{ctl}$
- Model 3: $(HD_{trt} - SHAM_{trt}) - (HD_{ctl} - SHAM_{ctl})$

where SHAM and HD are the means for sham sites and drug treated/HD-exposed sites, respectively, for the treatment (trt) or control (ctl) group indicated by the subscript. In addition, the relative response for each group and percent reduction in response for drug treated groups compared to control were calculated as

$$\text{Relative Response} = 100 * (HD - SHAM)/ SHAM$$

$$\text{Percent Change} = \frac{100 * [(HD_{trt} - SHAM_{trt})/ SHAM_{trt} - (HD_{ctl} - SHAM_{ctl})/ SHAM_{ctl}].}{[(HD_{ctl} - SHAM_{ctl})/ SHAM_{ctl}]}$$

$$\text{Percent Change in Severity} = 100 * [(Severity_{trt} - Severity_{ctl})/Severity_{ctl}]$$

where Severity is the mean severity score for the treatment or control group indicated by the subscript and the other abbreviations are defined above.

Results

Table 1 presents summary statistics and Fisher's Exact Test significance results for the histopath markers on HD-exposed sites for each drug treatment group. Notice that for intracellular edema and pustular epidermitis, no severity scores greater than 1 were reported, so that the mean severity score is equivalent to the percent incidence. The Fisher's Exact tests indicated that 1) animals treated with DEX had a significantly lower incidence of microblisters and pustular epidermitis than HD-exposed control animals and 2) animals treated with HC had a significantly lower incidence of pustular epidermitis than the HD-exposed control animals. No other comparisons were statistically significant.

Table 2 presents descriptive statistics for the average tissue weights at the sham and HD exposed sites in the drug treatment and control groups. Tables 3 and 4 present the results of the statistical analysis of tissue weight data. Model 1 tested for differences in tissue weights at sham sites between the drug treatment and control groups. The average tissue weight of sham sites on animals treated with DEX was significantly less than that of the sham sites on control animals, as shown in Table 3. This indicates that Dexamethasone had a systemic effect on tissue weight. The tissue weights for the sham sites treated with the other three compounds were not significantly different from the controls. Model 3 was fitted to examine the effects of drug treatment on HD-exposed sites after adjusting for differences in sham site responses. A statistically significant increase in tissue weights between HD-exposed sites and sham sites was observed for the control, DEX, HC, and OLV treatments, but not for the IND treatment, as summarized in Table 4. As indicated in the last three columns of Table 3, the weight shifts in the drug treatment groups were all significantly less than that observed in the control group.

Conclusions

The analysis of histopath markers indicated that 1) animals treated with DEX had a significantly lower incidence of microblisters and pustular epidermitis than HD-exposed control animals and 2) animals treated with HC had a significantly lower incidence of pustular epidermitis than the HD-exposed control animals.

A statistically significant increase in tissue weights between HD-exposed sites and sham sites was observed for the control, DEX, HC, and OLV treatments, but not for the IND treatment. The tissue weight increases in the drug treatment groups were all significantly less than that observed in the control group.

Table 1. Descriptive Statistics for Epidermal Necrosis (EN), Follicular Necrosis (FN), Intracellular Edema (IE), Microvesication (MV), and Pustular Epidermitis (PE) Histopath Markers in HD-Exposed Sites

Group	Summary Statistic	EN	FN	IE	MV	PE
Control	Number of Sites	16	16	16	16	16
	Mean Severity Score	3.25	1.44	0.00	0.94	0.38
	SD Severity Score	0.93	0.73	0.00	0.57	0.50
	Percent Change in Severity[1] (%)	NA	NA	NA	NA	NA
	Percent Incidence (%)	100	87.5	0	81.25	37.5
Dexamethasone	Number of Sites	20	20	20	20	20
	Mean Severity Score	2.50	1.20	0.05	0.35	0.05
	SD Severity Score	1.10	0.77	0.22	0.49	0.22
	Percent Change in Severity[1] (%)	-23.08	-16.52	NA	-62.67	-86.67
	Percent Incidence (%)	95	80	5	35*	5*
Hydrocortisone	Number of Sites	20	20	20	20	20
	Mean Severity Score	3.00	1.60	0.00	0.70	0.00
	SD Severity Score	1.08	0.68	0.00	0.73	0.00
	Percent Change in Severity[1] (%)	-7.69	11.30	NA	-25.33	-100.0
	Percent Incidence (%)	100	95	0	55	0*
Indomethacin	Number of Sites	20	20	20	20	20
	Mean Severity Score	2.90	1.30	0.20	1.05	0.25
	SD Severity Score	1.17	0.86	0.41	0.83	0.44
	Percent Change in Severity[1] (%)	-10.77	-9.57	NA	12.00	-33.33
	Percent Incidence (%)	95	80	20	75	25
Olvanil	Number of Sites	20	20	20	20	20
	Mean Severity Score	3.20	1.50	0.00	0.90	0.25
	SD Severity Score	0.95	0.76	0.00	0.79	0.44
	Percent Change in Severity[1] (%)	-1.54	4.35	NA	-4.00	-33.33
	Percent Incidence (%)	100	90	0	70	25

[1] Percent Change in Severity = 100 * [($Severity_{trt}$ – $Severity_{ctl}$)/$Severity_{ctl}$]. Note that the percent change does not apply to the control group, nor can it be calculated for IE, where the control group mean is 0.00.

*** Indicates percent incidence in drug treated group was significantly less than that observed in the control group, at the 0.05 level of significance.**

Table 2. Descriptive Statistics for Tissue Weights in HD-Exposed and Sham Sites

Group	Treatment	Exposure	Number of Animals	Tissue Weight (g)			
				Mean	SD	Relative Response[1]	Percent Change[2]
Control	None	Sham	8	0.081	0.011	32.46	0.00
	None	HD	8	0.107	0.015		
Dexamethasone	None	Sham	10	0.063	0.008	19.56	-39.74
	Dexamethasone	HD	10	0.076	0.012		
Hydrocortisone	None	Sham	10	0.072	0.010	10.61	-67.31
	Hydrocortisone	HD	10	0.079	0.012		
Indomethacin	None	Sham	10	0.083	0.012	7.33	-77.43
	Indomethacin	HD	10	0.089	0.015		
Olvanil	None	Sham	10	0.081	0.013	13.33	-58.93
	Olvanil	HD	10	0.092	0.015		

[1] Relative Response = 100 * (HD – SHAM)/ SHAM.
[2] Percent Change = 100 * $[(HD_{trt} - SHAM_{trt})/ SHAM_{trt} - (HD_{ctl} - SHAM_{ctl})/ SHAM_{ctl}]/[(HD_{ctl} - SHAM_{ctl})/ SHAM_{ctl}]$.

Table 3. Model Estimated Differences in Mean Tissue Weights Between Drug Treated and Control Groups

Model 1 Results				Model Chosen[1]	Final Model Results		
Comparison	Estimated Difference (sham sites)	SE	p-Value		Estimated Difference Due to Drug Treatment	SE	p-Value
DEX vs Control	-0.018	0.005	0.006	3	-0.014	0.005	0.049
HC vs Control	-0.009	0.005	0.239		-0.019	0.005	0.005
IND vs Control	0.002	0.005	0.988		-0.020	0.005	0.002
OLV vs Control	0.000	0.005	1.000		-0.015	0.005	0.025

[1] Model 2 was not required for this analysis.

Table 4. Estimated Difference in Tissue Weights between HD-Exposed Sites and Sham Sites within Each Drug Treatment Group

Group	Estimated Difference (HD – Sham)	Standard Error	p-Value (T-Test)
Control	0.026	0.004	<0.001
DEX	0.012	0.004	0.002
HC	0.008	0.004	0.044
IND	0.006	0.004	0.105
OLV	0.011	0.004	0.005

APPENDIX A:

LISTINGS OF ANALYSIS DATASETS

MREF Task 41
Histopath Data from SAS dataset created by SAS Access from Microsoft Excel File
Run 2 Tx Valid Histo & Wt Results.xls

Obs	DoseDate	Animal ID	Site	Tx	ICDNO	ICD	Treatment	Tissue Weight	EN	MV	FN	PE	IE
1	07/13/98	1	A	HD	2086	Indomethacin	Indomethacin	0.0928	3	1	1	0	1
2	07/13/98	1	B	Sham	2086	Indomethacin	None	0.0841	0	0	0	0	0
3	07/13/98	1	C	Sham	2086	Indomethacin	None	0.0899	0	0	0	0	0
4	07/13/98	1	D	HD	2086	Indomethacin	Indomethacin	0.0819	3	2	0	0	0
5	07/13/98	2	A	HD	2086	Indomethacin	Indomethacin	0.1056	4	1	1	1	0
6	07/13/98	2	B	Sham	2086	Indomethacin	None	0.0722	0	0	0	0	0
7	07/13/98	2	C	Sham	2086	Indomethacin	None	0.1178	0	0	0	0	0
8	07/13/98	2	D	HD	2086	Indomethacin	Indomethacin	0.1014	2	0	1	0	0
9	07/13/98	3	A	HD	2086	Indomethacin	Indomethacin	0.0888	2	1	0	0	0
10	07/13/98	3	B	Sham	2086	Indomethacin	None	0.0797	0	0	0	0	0
11	07/13/98	3	C	Sham	2086	Indomethacin	None	0.0533	0	0	0	0	0
12	07/13/98	3	D	HD	2086	Indomethacin	Indomethacin	0.0751	2	0	1	0	0
13	07/13/98	4	A	HD	2842	Hydrocortisone	Hydrocortisone	0.0727	4	1	1	0	0
14	07/13/98	4	B	Sham	2842	Hydrocortisone	None	0.0649	0	0	0	0	0
15	07/13/98	4	C	Sham	2842	Hydrocortisone	None	0.0502	0	0	0	0	0
16	07/13/98	4	D	HD	2842	Hydrocortisone	Hydrocortisone	0.0797	4	1	1	0	0
17	07/13/98	5	A	HD	2842	Hydrocortisone	Hydrocortisone	0.0664	1	0	1	0	0
18	07/13/98	5	B	Sham	2842	Hydrocortisone	None	0.0707	0	0	0	0	0
19	07/13/98	5	C	Sham	2842	Hydrocortisone	None	0.0641	0	0	0	0	0
20	07/13/98	5	D	HD	2842	Hydrocortisone	Hydrocortisone	0.0746	3	1	1	0	0
21	07/13/98	6	A	HD	2842	Hydrocortisone	Hydrocortisone	0.0900	1	0	1	0	0
22	07/13/98	6	B	Sham	2842	Hydrocortisone	None	0.0709	0	0	0	0	0
23	07/13/98	6	C	Sham	2842	Hydrocortisone	None	0.0574	0	0	0	0	0
24	07/13/98	6	D	HD	2842	Hydrocortisone	Hydrocortisone	0.0761	3	0	1	0	0
25	07/13/98	7	A	HD	2723	Olvanil	Olvanil	0.1097	4	2	1	0	0
26	07/13/98	7	B	Sham	2723	Olvanil	None	0.1085	0	0	0	0	0
27	07/13/98	7	C	Sham	2723	Olvanil	None	0.0895	0	0	0	0	0
28	07/13/98	7	D	HD	2723	Olvanil	Olvanil	0.1024	3	1	1	0	0
29	07/13/98	8	A	HD	2723	Olvanil	Olvanil	0.1048	2	0	0	0	0
30	07/13/98	8	B	Sham	2723	Olvanil	None	0.0880	0	0	0	0	0
31	07/13/98	8	C	Sham	2723	Olvanil	None	0.0852	0	0	0	0	0
32	07/13/98	8	D	HD	2723	Olvanil	Olvanil	0.1137	2	1	1	0	0
33	07/13/98	9	A	HD	2723	Olvanil	Olvanil	0.0888	2	0	1	0	0
34	07/13/98	9	B	Sham	2723	Olvanil	None	0.1064	0	0	0	0	0
35	07/13/98	9	C	Sham	2723	Olvanil	None	0.0738	0	0	0	0	0
36	07/13/98	9	D	HD	2723	Olvanil	Olvanil	0.1030	2	1	1	0	0
37	07/13/98	10	A	HD	2845	Dexamethasone	Dexamethasone	0.0798	3	1	1	0	0
38	07/13/98	10	B	Sham	2845	Dexamethasone	None	0.0671	0	0	0	0	0
39	07/13/98	10	C	Sham	2845	Dexamethasone	None	0.0607	0	0	0	0	0
40	07/13/98	10	D	HD	2845	Dexamethasone	Dexamethasone	0.0757	3	1	1	0	0
41	07/13/98	11	A	HD	2845	Dexamethasone	Dexamethasone	0.0719	2	0	1	0	0
42	07/13/98	11	B	Sham	2845	Dexamethasone	None	0.0612	0	0	0	0	0
43	07/13/98	11	C	Sham	2845	Dexamethasone	None	0.0634	0	0	0	0	0
44	07/13/98	11	D	HD	2845	Dexamethasone	Dexamethasone	0.0478	2	0	0	0	0
45	07/13/98	12	A	HD	2845	Dexamethasone	Dexamethasone	0.0764	2	1	1	0	0
46	07/13/98	12	B	Sham	2845	Dexamethasone	None	0.0705	0	0	0	0	0
47	07/13/98	12	C	Sham	2845	Dexamethasone	None	0.0675	0	0	0	0	0
48	07/13/98	12	D	HD	2845	Dexamethasone	Dexamethasone	0.0761	2	0	1	0	0
49	07/13/98	13	A	HD	0	Control	None	0.1008	4	1	1	1	0
50	07/13/98	13	B	Sham	0	Control	None	0.0667	0	0	0	0	0
51	07/13/98	13	C	Sham	0	Control	None	0.0666	0	0	0	0	0
52	07/13/98	13	D	HD	0	Control	None	0.0788	2	0	0	0	0
53	07/13/98	14	A	HD	0	Control	None	0.1171	3	1	2	1	0
54	07/13/98	14	B	Sham	0	Control	None	0.0764	0	0	0	0	0
55	07/13/98	14	C	Sham	0	Control	None	0.0665	0	0	0	0	0
56	07/13/98	14	D	HD	0	Control	None	0.1041	2	1	1	0	0
57	07/13/98	15	A	HD	0	Control	None	0.1298	3	1	2	1	0
58	07/13/98	15	B	Sham	0	Control	None	0.0926	0	0	0	0	0
59	07/13/98	15	C	Sham	0	Control	None	0.0927	0	0	0	0	0
60	07/13/98	15	D	HD	0	Control	None	0.1035	3	1	1	0	0
61	07/16/98	1	A	HD	2086	Indomethacin	Indomethacin	0.0944	3	1	1	1	0
62	07/16/98	1	B	Sham	2086	Indomethacin	None	0.0907	0	0	0	0	0
63	07/16/98	1	C	Sham	2086	Indomethacin	None	0.0692	1	0	0	0	0
64	07/16/98	1	D	HD	2086	Indomethacin	Indomethacin	0.0732	2	1	1	0	0

65	07/16/98	2	A	HD	2086	Indomethacin	Indomethacin	0.1158	2	1	2	0	0
66	07/16/98	2	B	Sham	2086	Indomethacin	None	0.1030	0	0	0	0	0
67	07/16/98	2	C	Sham	2086	Indomethacin	None	0.1006	0	0	0	0	0
68	07/16/98	2	D	HD	2086	Indomethacin	Indomethacin	0.1020	3	1	2	0	0
69	07/16/98	3	A	HD	2086	Indomethacin	Indomethacin	0.0613	4	1	2	0	0
70	07/16/98	3	B	Sham	2086	Indomethacin	None	0.0614	0	0	0	0	0
71	07/16/98	3	C	Sham	2086	Indomethacin	None	0.0829	0	0	0	0	0

Histopath Data from SAS dataset created by SAS Access from Microsoft Excel file
Run 2 Tx Valid Histo & Wt Results.xls

Obs	DoseDate	Animal ID	Site	Tx	ICDNO	ICD	Treatment	Tissue Weight	EN	MV	FN	PE	IE
72	07/16/98	3	D	HD	2086	Indomethacin	Indomethacin	0.0747	4	2	2	0	0
73	07/16/98	4	A	HD	2842	Hydrocortisone	Hydrocortisone	0.0663	3	0	2	0	0
74	07/16/98	4	B	Sham	2842	Hydrocortisone	None	0.0891	0	0	0	0	0
75	07/16/98	4	C	Sham	2842	Hydrocortisone	None	0.0780	0	0	0	0	0
76	07/16/98	4	D	HD	2842	Hydrocortisone	Hydrocortisone	0.0635	4	2	2	0	0
77	07/16/98	5	A	HD	2842	Hydrocortisone	Hydrocortisone	0.0775	2	0	0	0	0
78	07/16/98	5	B	Sham	2842	Hydrocortisone	None	0.0795	0	0	0	0	0
79	07/16/98	5	C	Sham	2842	Hydrocortisone	None	0.0783	0	0	0	0	0
80	07/16/98	5	D	HD	2842	Hydrocortisone	Hydrocortisone	0.0877	2	1	2	0	0
81	07/16/98	6	A	HD	2842	Hydrocortisone	Hydrocortisone	0.0688	4	2	2	0	0
82	07/16/98	6	B	Sham	2842	Hydrocortisone	None	0.0709	0	0	0	0	0
83	07/16/98	6	C	Sham	2842	Hydrocortisone	None	0.0575	0	0	0	0	0
84	07/16/98	6	D	HD	2842	Hydrocortisone	Hydrocortisone	0.0588	4	1	2	0	0
85	07/16/98	7	A	HD	2723	Olvanil	Olvanil	0.0750	4	1	2	0	0
86	07/16/98	7	B	Sham	2723	Olvanil	None	0.0602	0	0	0	0	0
87	07/16/98	7	C	Sham	2723	Olvanil	None	0.0637	0	0	0	0	0
88	07/16/98	7	D	HD	2723	Olvanil	Olvanil	0.0687	4	2	3	0	0
89	07/16/98	8	A	HD	2723	Olvanil	Olvanil	0.1215	4	1	2	1	0
90	07/16/98	8	B	Sham	2723	Olvanil	None	0.0732	0	0	0	0	0
91	07/16/98	8	C	Sham	2723	Olvanil	None	0.0679	0	0	0	0	0
92	07/16/98	8	D	HD	2723	Olvanil	Olvanil	0.0822	2	0	0	0	0
93	07/16/98	9	A	HD	2723	Olvanil	Olvanil	0.0735	4	0	2	1	0
94	07/16/98	9	B	Sham	2723	Olvanil	None	0.0589	0	0	0	0	0
95	07/16/98	9	C	Sham	2723	Olvanil	None	0.0813	0	0	0	0	0
96	07/16/98	9	D	HD	2723	Olvanil	Olvanil	0.0771	4	1	2	0	0
97	07/16/98	10	A	HD	2845	Dexamethasone	Dexamethasone	0.0598	2	0	2	0	0
98	07/16/98	10	B	Sham	2845	Dexamethasone	None	0.0763	0	0	0	0	0
99	07/16/98	10	C	Sham	2845	Dexamethasone	None	0.0722	0	0	0	0	0
100	07/16/98	10	D	HD	2845	Dexamethasone	Dexamethasone	0.0564	2	0	1	0	0
101	07/16/98	11	A	HD	2845	Dexamethasone	Dexamethasone	0.0720	4	1	2	0	0
102	07/16/98	11	B	Sham	2845	Dexamethasone	None	0.0502	0	0	0	0	0
103	07/16/98	11	C	Sham	2845	Dexamethasone	None	0.0556	0	0	0	0	0
104	07/16/98	11	D	HD	2845	Dexamethasone	Dexamethasone	0.0577	2	0	0	0	0
105	07/16/98	12	A	HD	2845	Dexamethasone	Dexamethasone	0.0846	2	1	2	0	0
106	07/16/98	12	B	Sham	2845	Dexamethasone	None	0.0708	0	0	0	0	0
107	07/16/98	12	C	Sham	2845	Dexamethasone	None	0.0645	0	0	0	0	0
108	07/16/98	12	D	HD	2845	Dexamethasone	Dexamethasone	0.0911	1	0	0	0	0
109	07/16/98	13	A	HD	0	Control	None	0.1132	4	1	2	0	0
110	07/16/98	13	B	Sham	0	Control	None	0.0726	0	0	0	0	0
111	07/16/98	13	C	Sham	0	Control	None	0.0848	0	0	0	0	0
112	07/16/98	13	D	HD	0	Control	None	0.1149	3	1	1	0	0
113	07/16/98	14	A	HD	0	Control	None	0.1204	4	2	2	0	0
114	07/16/98	14	B	Sham	0	Control	None	0.0919	0	0	0	0	0
115	07/16/98	14	C	Sham	0	Control	None	0.0923	0	0	0	0	0
116	07/16/98	14	D	HD	0	Control	None	0.1075	4	1	2	0	0
117	07/16/98	15	A	HD	0	Control	None	0.1305	4	2	2	1	0
118	07/16/98	15	B	Sham	0	Control	None	0.0960	0	0	0	0	0
119	07/16/98	15	C	Sham	0	Control	None	0.0964	0	0	0	0	0
120	07/16/98	15	D	HD	0	Control	None	0.1322	4	0	2	0	0
121	07/20/98	1	A	HD	2086	Indomethacin	Indomethacin	0.0734	4	2	2	0	1
122	07/20/98	1	B	Sham	2086	Indomethacin	None	0.0816	0	0	0	0	0
123	07/20/98	1	C	Sham	2086	Indomethacin	None	0.0845	0	0	0	0	0
124	07/20/98	1	D	HD	2086	Indomethacin	Indomethacin	0.0831	1	0	0	0	1
125	07/20/98	2	A	HD	2086	Indomethacin	Indomethacin	0.1289	3	2	1	0	1
126	07/20/98	2	B	Sham	2086	Indomethacin	None	0.0781	0	0	0	0	0
127	07/20/98	2	C	Sham	2086	Indomethacin	None	0.0962	0	0	0	0	0
128	07/20/98	2	D	HD	2086	Indomethacin	Indomethacin	0.0974	4	0	2	0	0
129	07/20/98	3	A	HD	2086	Indomethacin	Indomethacin	0.1026	4	3	3	1	0
130	07/20/98	3	B	Sham	2086	Indomethacin	None	0.1051	0	0	0	0	0
131	07/20/98	3	C	Sham	2086	Indomethacin	None	0.0797	0	0	0	0	0
132	07/20/98	3	D	HD	2086	Indomethacin	Indomethacin	0.0799	0	0	0	0	0
133	07/20/98	4	A	HD	2086	Indomethacin	Indomethacin	0.0627	4	1	2	1	0
134	07/20/98	4	B	Sham	2086	Indomethacin	None	0.0533	0	0	0	0	0
135	07/20/98	4	C	Sham	2086	Indomethacin	None	0.0751	0	0	0	0	0

136	07/20/98	4	D	HD	2086	Indomethacin	Indomethacin	0.0849	4	1	2	1	0
137	07/20/98	5	A	HD	2842	Hydrocortisone	Hydrocortisone	0.0670	2	0	1	0	0
138	07/20/98	5	B	Sham	2842	Hydrocortisone	None	0.0679	0	0	0	0	0
139	07/20/98	5	C	Sham	2842	Hydrocortisone	None	0.0576	0	0	0	0	0
140	07/20/98	5	D	HD	2842	Hydrocortisone	Hydrocortisone	0.0815	2	0	2	0	0
141	07/20/98	6	A	HD	2842	Hydrocortisone	Hydrocortisone	0.0990	4	1	2	0	0
142	07/20/98	6	B	Sham	2842	Hydrocortisone	None	0.0893	0	0	0	0	0

Histopath Data from SAS dataset created by SAS Access from Microsoft Excel file
Run 2 Tx Valid Histo & Wt Results.xls

Obs	DoseDate	Animal ID	Site	Tx	ICDNO	ICD	Treatment	Tissue Weight	EN	MV	FN	PE	IE
143	07/20/98	6	C	Sham	2842	Hydrocortisone	None	0.0900	0	0	0	0	0
144	07/20/98	6	D	HD	2842	Hydrocortisone	Hydrocortisone	0.1035	4	0	3	0	0
145	07/20/98	7	A	HD	2842	Hydrocortisone	Hydrocortisone	0.0957	3	1	2	0	0
146	07/20/98	7	B	Sham	2842	Hydrocortisone	None	0.0783	0	0	0	0	0
147	07/20/98	7	C	Sham	2842	Hydrocortisone	None	0.0623	0	0	0	0	0
148	07/20/98	7	D	HD	2842	Hydrocortisone	Hydrocortisone	0.0755	4	2	2	0	0
149	07/20/98	8	A	HD	2842	Hydrocortisone	Hydrocortisone	0.0953	4	1	2	0	0
150	07/20/98	8	B	Sham	2842	Hydrocortisone	None	0.0748	0	0	0	0	0
151	07/20/98	8	C	Sham	2842	Hydrocortisone	None	0.0826	0	0	0	0	0
152	07/20/98	8	D	HD	2842	Hydrocortisone	Hydrocortisone	0.0869	2	0	2	0	0
153	07/20/98	9	A	HD	2723	Olvanil	Olvanil	0.1016	4	1	2	0	0
154	07/20/98	9	B	Sham	2723	Olvanil	None	0.0854	0	0	0	0	0
155	07/20/98	9	C	Sham	2723	Olvanil	None	0.0684	0	0	0	0	0
156	07/20/98	9	D	HD	2723	Olvanil	Olvanil	0.1131	4	1	2	1	0
157	07/20/98	10	A	HD	2723	Olvanil	Olvanil	0.0679	4	1	2	0	0
158	07/20/98	10	B	Sham	2723	Olvanil	None	0.0764	0	0	0	0	0
159	07/20/98	10	C	Sham	2723	Olvanil	None	0.0832	0	0	0	0	0
160	07/20/98	10	D	HD	2723	Olvanil	Olvanil	0.0974	4	3	2	1	0
161	07/20/98	11	A	HD	2723	Olvanil	Olvanil	0.1036	4	1	2	0	0
162	07/20/98	11	B	Sham	2723	Olvanil	None	0.0919	0	0	0	0	0
163	07/20/98	11	C	Sham	2723	Olvanil	None	0.1152	0	0	0	0	0
164	07/20/98	11	D	HD	2723	Olvanil	Olvanil	0.0896	2	0	1	0	0
165	07/20/98	12	A	HD	2723	Olvanil	Olvanil	0.0812	3	0	2	0	0
166	07/20/98	12	B	Sham	2723	Olvanil	None	0.0767	0	0	0	0	0
167	07/20/98	12	C	Sham	2723	Olvanil	None	0.0739	0	0	0	0	0
168	07/20/98	12	D	HD	2723	Olvanil	Olvanil	0.0699	2	1	1	1	0
169	07/20/98	13	A	HD	2845	Dexamethasone	Dexamethasone	0.1094	4	1	2	1	0
170	07/20/98	13	B	Sham	2845	Dexamethasone	None	0.0550	0	0	0	0	0
171	07/20/98	13	C	Sham	2845	Dexamethasone	None	0.0863	0	0	0	0	0
172	07/20/98	13	D	HD	2845	Dexamethasone	Dexamethasone	0.0681	0	0	0	0	0
173	07/20/98	14	A	HD	2845	Dexamethasone	Dexamethasone	0.1012	4	1	2	0	0
174	07/20/98	14	B	Sham	2845	Dexamethasone	None	0.0650	0	0	0	0	0
175	07/20/98	14	C	Sham	2845	Dexamethasone	None	0.0642	0	0	0	0	0
176	07/20/98	14	D	HD	2845	Dexamethasone	Dexamethasone	0.0828	3	0	2	0	0
177	07/20/98	15	A	HD	2845	Dexamethasone	Dexamethasone	0.0554	2	0	1	0	1
178	07/20/98	15	B	Sham	2845	Dexamethasone	None	0.0577	0	0	0	0	0
179	07/20/98	15	C	Sham	2845	Dexamethasone	None	0.0510	0	0	0	0	0
180	07/20/98	15	D	HD	2845	Dexamethasone	Dexamethasone	0.0837	2	0	1	0	0
181	07/20/98	16	A	HD	2845	Dexamethasone	Dexamethasone	0.1068	4	0	2	0	0
182	07/20/98	16	B	Sham	2845	Dexamethasone	None	0.0527	0	0	0	0	0
183	07/20/98	16	C	Sham	2845	Dexamethasone	None	0.0538	0	0	0	0	0
184	07/20/98	16	D	HD	2845	Dexamethasone	Dexamethasone	0.0566	4	0	2	0	0
185	07/20/98	17	A	HD	0	Control	None	0.1057	4	1	2	0	0
186	07/20/98	17	B	Sham	0	Control	None	0.1017	0	0	0	0	0
187	07/20/98	17	C	Sham	0	Control	None	0.0581	0	0	0	0	0
188	07/20/98	17	D	HD	0	Control	None	0.0734	1	0	0	0	0
189	07/20/98	18	A	HD	0	Control	None	0.0968	4	1	2	1	0
190	07/20/98	18	B	Sham	0	Control	None	0.0744	0	0	0	0	0
191	07/20/98	18	C	Sham	0	Control	None	0.0666	0	0	0	0	0
192	07/20/98	18	D	HD	0	Control	None	0.0884	3	1	1	1	0

APPENDIX K

Addendum to Statistical Analyses

Battelle
. . . Putting Technology To Work

Project Number G1555-B41ASTAT (3104)

Internal Distribution

Lee/Dept. Files
BK Pierce
BJ Wood
JR Holdcraft
NA Niemuth
RMO

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Date August 7, 2000

To **Carol Sabourin**

From Nancy Niemuth

Subject **Addendum to Statistical Analysis of Study G1555-41A MREF Task 95-41**

The attached tables contain the mean and standard error of the relative tissue weights for MREF Task 95-41 Modules I, II, II Histopath, and III. Statistical comparisons of relative tissue weights between post-exposure times (Module I) or between drug treated and control groups (Modules II and III) were conducted using t-tests within analysis of variance (ANOVA) models, consistent with the methodology used for the analysis of relative ear weights in MREF Task 95-43.

Note that the mean relative tissue weights are very close to but not exactly the same as those presented in the statistical analysis reports for MREF Task 95-41 Modules I, II, II Histopath, and III. The relative tissue weight in the previous statistical analysis reports was calculated using the average tissue weight across all animals for the sham sites and for the HD-treated sites, while the relative tissue weight in the attached tables was calculated for each animal and then averaged. These calculation methods are not equivalent and thus give slightly different results.

Please call me at (614)424-3231 if you have any questions.

NAN:llj
Attachments

For Review and Approval

	Name	Initials	Date
Originator	Nancy Niemuth	N	8/7/00
Concurrence	Jennifer Holdcraft	JRH	8/7/00
	Brandon Wood	BJW	8/7/00
Approved	Bill Rosebrough	WRR	8/7/00

Sent Via: Interoffice Mail

Table 1. Mean and SE for Module I Relative Tissue Weights by Time Post-Exposure

Module I - Relative Tissue Weight		
Time Post-Exposure	**Mean**	**SE**
2 hr	0.650 [a]	2.187
6 hr	9.188 [a]	3.981
24 hr	33.958	4.879

(a) Mean relative tissue weight is statistically less than that observed for the 24 hour group based on ANOVA t-test conducted at the 5 percent level

Table 2. Mean, SE, and Percent Change for Module II Relative Tissue Weights by Treatment Group

Relative Tissue Weight - Module II			
Group	**Mean**	**SE**	**Percent Change Relative to Control (%)**
Control	44.427	5.653	NA
Olvanil	20.647 [a]	7.948	-54
Dexamethasone	23.401 [a]	4.987	-47

(a) Mean relative ear weight is statistically less than that observed for the HD control group based on ANOVA t-test conducted at the 5 percent level

Table 3. Mean, SE, and Percent Change for Module II Histopath Relative Tissue Weights by Treatment Group

Module II Histopath - Relative Tissue Weight			
Group	**Mean**	**SE**	**Percent Change Relative to Control (%)**
Control	33.005	4.647	0.00
Dexamethasone	20.838	6.848	-36.86
Hydrocortisone	11.700 [a]	5.073	-64.55
Indomethacin	7.638 [a]	3.786	-76.86
Olvanil	14.427 [a]	5.374	-56.29

(a) Mean relative tissue weight is statistically less than that observed for the HD control group based on ANOVA t-test conducted at the 5 percent level

Table 4. Mean, SE, and Percent Change for Module III Relative Tissue Weights for each Treatment Group at 6 and 24 Hours Post-Exposure

Relative Tissue Weight - Module III				
Exposure Time	**Group**	**Mean**	**SE**	**Percent Change Relative to Control (%)**
6	Ethanol	57.833	9.766	NA
	Indomethacin	24.683 [a]	6.496	-57
	Olvanil	50.488	7.130	-13
	Hydrocortisone	13.358 [a]	1.824	-77
	Dexamethasone	12.404 [a]	0.977	-79
24	Ethanol	158.391	5.004	NA
	Indomethacin	101.780 [a]	10.406	-36
	Olvanil	131.650	15.872	-17
	Hydrocortisone	135.869	12.147	-14
	Dexamethasone	143.327	11.614	-10

(a) Mean relative ear weight is statistically less than that observed for the HD control group based on ANOVA t-test conducted at the 5 percent level